THE EFFECTS OF PARITY AND THE MENSTRUAL CYCLE ON THE NORMAL MAMMARY GLAND AND THEIR POSSIBLE RELATIONSHIP TO MALIGNANT CHANGE

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DECLARATION

In accordance with the statutes of the University of Edinburgh, I declare that the preparation and writing of this thesis has been carried out by myself.

The research described in this thesis was carried out by myself except where the contribution of others is acknowledged.

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ABSTRACT

The effect of the menstrual cycle on the breast was investigated in nulliparous and parous women.

Breast volume was measured by water displacement in four nulliparous women. Volumes were least in the week after menstruation, and increased by 20% to a maximum just before menstruation.

From 174 women of reproductive age 212 specimens of normal breast tissue were obtained during biopsy or mammoplasty. Menstrual and reproductive histories were taken, and plasma progesterone and oestradiol concentrations were measured. Histological examination of lobules, ductules, and ductule epithelium was carried out. There was much variation between specimens. Parous women had a greater density of lobules than nulliparae, and parity caused a change in ductule epithelium: clear basal cells were much more numerous among nulliparous than parous women. Increased epithelial height during the luteal phase of the menstrual cycle was noted among both parous women and nulliparae, but the cycle produced no other changes. Lobule density was less within ten years of the menarche, but age was associated with no other lobular changes.

DNA synthesis by epithelial cells was studied in 52 specimens by organ culture with labelled thymidine followed by autoradiography. Among nulliparae there was no cyclical variation, but among parous women synthesis was greatest during the luteal phase of the cycle.

Synthesis of Immunoglobulin A was studied in 80 specimens by immunofluorescence and by in vitro culture with labelled amino acids followed by radioimmunoelectrophoresis. Among nulliparae there were

no cyclical variations, but among parous women synthesis was greatest during the luteal phase of the cycle.

It is suggested therefore that the nulliparous breast is less sensitive to progesterone than is the parous breast. Before first pregnancy oestrogen stimulation of mammary epithelium may be inadequately opposed by progesterone, and this may explain the increased incidence of breast cancer among nulliparae.

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PREVIOUSLY PUBLISHED WORK

Parts of the work described here have been published in the following papers:

Chapter 3: (1) Milligan D, Drife JO, Short RV. "Changes in breast volume during normal menstrual cycle and after oral contraceptives". British Medical Journal 1975 iv 494-6.

Chapter 4: (2) Short RV, Drife JO. "The aetiology of mammary cancer in man and animals". Symposia of the Zoological Society of London 1977 41 211-30

(3) Drife JO. "Evolution, menstruation and breast cancer". In Commentaries of Research in Breast Disease, 1 ed RD Bulbrook and D Jane Taylor, p 1-23 New York, Alan R Liss 1979.

Chapter 5: (4) Masters JRW, Drife JO, Scarisbrick JJ. "Cyclic variation of DNA synthesis in human breast epithelium". Journal of the National Cancer Institute 1977 58 1263-5.

Chapter 6: (5) Drife JO, McClelland DBL, Pryde AA, Roberts MM, Smith II. "Immunoglobulin synthesis in the 'resting' breast". British Medical Journal 1976 ii 503-6.

Chapter 7: (6) Drife JO. "Breast cancer, pregnancy and the pill" British Medical Journal 1981 283 778-9.

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Chapter 1

INTRODUCTION

The research described in this thesis aimed at defining the structural and functional changes that occur in the human female mammary gland as a result of the menstrual cycle, both in nulliparous and in parous women. The reasons for undertaking this research are as follows.

Although the human mammary gland has been the subject of a great deal of research, most of the investigation has concentrated on either the gland which has undergone malignant change or the gland which is the seat of benign disease. There has been relatively little investigation of the healthy, non-lactating gland, and controversy still exists on the most funadmental questions about its structure. In particular, there is no consensus on the question of whether or not changes occur in the structure of the breast as a result of the menstrual cycle. Although several investigations have addressed this question, their conclusions differ markedly from one another. (The investigations are discussed in detail in Chapter 2). On reviewing these studies, it became clear that none had examined separately nulliparous and parous women. The first hypothesis to be tested, therefore, was that the breast of the nulliparous woman responds differently to the menstrual cycle from that of the parous woman. In other words, the experience of pregnancy may alter the mammary gland's response to the menstrual cycle. This may be the reason that previous investigators of breast histology had failed to agree on the effects of the cycle.

Since an understanding of the normal is a prerequisite for studying the abnormal, clarification of this point would be of value in further investigation of the diseased gland. However, the experience of

pregnancy is of particular relevance to the mammary gland. Nulliparous women have a higher risk of developing breast cancer than do parous women. Large-scale epidemiological investigations published early in the 1970s confirmed this fact, and showed also that the earlier in her life a woman experiences her first full-term pregnancy, the smaller her risk of developing breast cancer later in her life. (These investigations are discussed in detail in Chapter 7.) Thus the experience of one full-term pregnancy alters the susceptibility of the breast to the development of malignant change.

For a variety of reasons - discussed in Chapter 7 - ovarian hormones are now thought to be implicated in the development of malignant change in the breast. The importance, for example, of the age at menarche and the age at menopause is now recognised as partly determining a woman's risk of developing breast cancer. Therefore an understanding of the response of the normal breast to the hormonal changes of the menstrual cycle becomes particularly important. Breast cancer is the commonest malignancy affecting women, and some 10,000 British women each year are affected by the condition. The results of treatment have not improved over the years, and more attention is now being paid to the causes of the disease, in the hope of identifying avoidable or preventable factors.

If the hypothesis (that the breast before first pregnancy differs in its response to the menstrual cycle from the breast of a parous woman) appears tenable, then further hypotheses could be developed with direct clinical relevance. For example, if the breast of a nulliparous woman responds to the cycle, but no cyclical changes

occur among parous women, then it could be suggested that the cyclical changes among nulliparae represent a "stress" to the breast, which could be connected with its increased tendency to develop malignant change. If such changes could be abolished (for example, by continuous use of oral contraceptives) then the increased risk of breast cancer among nulliparous women might be reduced.

Before such testable theories can be elaborated in any detail, the first step is to find out whether cyclical changes occur in the breast, and then whether they occur equally in nulliparous and parous women. The changes investigated fall under four headings: macroscopic, histological, DNA-synthesis, and immunological. Each is described in a separate chapter in this thesis.

In Chapter 2 previous investigations of the structural development of the breast and its endocrine control are reviewed. In Chapter 3 an investigation of gross morphological changes during the cycle is described. Chapter 4 described the histological investigation of normal breast tissue from 174 women. Chapter 5 describes organ culture work investigating DNA synthesis, and Chapter 6 describes work on in vitro immunoglobulin synthesis. In the final chapter, (7), the epidemiology of breast cancer is reviewed in the light of the results of this work, and a new hypothesis is put forward to explain the connection between ovarian hormones and breast cancer.

CHAPTER 2

NORMAL ANATOMICAL AND PHYSIOLOGICAL DEVELOPMENT OF THE HUMAN MAMMARY GLAND, AND ITS ENDOCRINE CONTROL

The female mammary gland consists of glandular tissue and ducts, surrounded by a stroma of fibrous tissue and fat. The relative proportions of these components vary depending on the stage of the woman's reproductive life. This variation occurs partly as a result of hormonal influences, the most important being the effects of ovarian steroids.

The first experiments on the effect of the ovary on the mammary gland were described early in this century. In 1914 Ott and Scott ground up bovine corpora lutea and injected them into rabbits. They noted that the glands increased in size and contained milk, and that under the microscope there was a marked increase in the number of glandular structures. The importance of the pituitary was also recognised (Ott, 1910). Since that time many hormones have been implicated in the control of breast development, and lactation. Most of the experimental work has been done on a variety of animal species and this is a potential source of confusion, though Anderson (1974) points out that there are an estimated 19,000 species in the class mammalia and investigation has been carried out in relatively few of these.

A Embryonic and fetal development

The "milk streaks" appear before the sixth week in the embryo, as two broad zones of skin on the antero-lateral aspects of the thorax and abdomen, characterised by taller epithelial cells and an increased vascularity of subepidermal tissue (Dawson 1934). The streaks disappear by the tenth week of fetal life, leaving only the nipple area, and at about the 15thweek this epithelium starts budding downwards. 15 - 20 cords of epithelial cells pass into the subjacent connective tissue

and after about the 20th week they become canalised: the ducts thus formed are said to have a wall consisting of a two-layered epithelium - an outer, basal layer of flattened cells and an inner layer of columnar cells next to the duct lumen. Mitotic figures are rare in fetal breast epithelium (Dawson 1934).

Embryonic mammary development appears to be independent of hormonal control (Vorherr 1974; Anderson 1974), though stimulation of the fetal breast by placental steroids does occur during the last trimester of pregnancy. Experimental work has been done on mouse embryos (Lasfargues and Murray 1959) by cultivation of the mammary apparatus in artificial media. Growth proceeded to the stage of secondary duct development without the addition of hormones, but growth and differentiation were enhanced by the addition of growth hormone or prolactin. Growth of the epithelium itself was affected only by growth hormone, while prolactin was said to affect differentiation. Steroid hormones were also tested, and the interesting observation was made that oestradiol stimulated the adipose tissue but not the epithelium. Progesterone promoted breakdown of ground substance and cortisol enhanced changes which were indicative of secretion. The effects of testosterone were investigated by Raynaud (1949), and this steroid is thought to promote the separation of the mammary bud from the epithelium - an event in rats and mice which occurs only in the male. No such separation occurs in the human, and there is no evidence for androgens having an effect on human embryonic breast tissue.

Later in fetal development, however, an inhibitory effect of androgens has been shown on mammary development in organ culture of

rat and mouse tissue (Ceriani 1970; Turkington and Topper 1967). Similar work has shown that various hormones are required for *in vitro* mammary growth: insulin, prolactin, aldosterone and progesterone. Ceriani (1970) has suggested that these hormones are not all necessary to development of all parts of the ductal "tree" - for example, only insulin is required for the main duct to develop, while the development of the smallest ductules requires all four hormones. Ceriani found that oestradiol-17B and growth hormone had no effect on fetal gland development *in vitro*.

Development of mammary tissue will proceed slowly in organ culture systems without the addition of any hormones. It is difficult to extrapolate these *in vitro* findings to the normal situation *in vivo*, and the fact that the effect of a hormone can be shown in an organ culture system does not, of course, mean that the effect is necessarily exerted in *vivo*. The lack of effect of oestradiol, however, would appear to indicate that this hormone does not play a significant part in pre-natal mammary development.

B Development from birth to puberty

According to Dawson (1934), at the time of birth an elementary system of ducts exists without lobular structures or true secreting tissue. However, Dabelow (1957) and Bassler (1970) describe "lobulo-alveolar structures resembling endvesicles" containing colostrum. These are seen in both sexes, and an elevation of the mammary discs is also seen. The end-vesicles are said to regress and are not seen in childhood. According to Vorherr (1974) the connective tissue of the breast at the time of birth consists of three layers: (1) a superficial

layer under the nipple, (2) an intermediate zone which surrounds the ducts and extends into peripheral connective tissue septa, and (3) an inner connective tissue layer which envelops the deepest epithelial elements. The significance of this layering is not explained, and it is not clear from the literature whether the connective tissue near the ducts is loose (as it is in the mature gland) compared to the remainder of the stroma.

There is histological evidence of stimulation of the glandular epithelium at the time of birth, and according to Vorherr (1974) the colostral secretion seen in some 90% of newborn infants is caused by the withdrawal of sex steroids allowing prolactin to stimulate the mammary epithelium. This stimulation is maximal at 4-7 days post-partum, and subsides within 3-4 weeks. However, Vorherr quotes no evidence to show that a rise in prolactin levels does in fact occur. Neonatal colostrum secretion occurs also in other species, such as rabbit, cat, goat, and horse (Tucker 1974) and Tucker points out that fetal glucocorticoids may be involved in its production as their levels rise around the time of parturition. The traditional view is that the secretion is stimulated by placental transfer of maternal hormones (Vaughan 1969): other tissues of the neonate also show evidence of hormonal stimulation which settles down soon after birth.

During the period from birth to puberty, only sufficient growth to keep pace with general body growth takes place. There are no lobules, and the two-layered structure of the duct wall is still seen. Normally there is no evidence of specific hormonal effects, but in cases of precocious puberty breast development occurs with stromal and glandular

development as in normal physiological puberty (Dawson 1934). Therefore the breast in childhood appears capable of responding to hormones, and the lack of breast development before puberty is a consequence only of low hormone levels.

C Puberty

Breast development is the first sign of puberty (Marshall 1970), beginning at a mean age of 11 and being completed at a mean age of 15½ (Marshall and Tanner 1974). The menarche occurs somewhat later: the age at menarche has been falling steadily over the last ten years but now seems to have stabilised at a mean of 13.2 years in Britain (Dann and Roberts 1973; Roberts and Dann 1975). (It is probable, though not proven, that this fall is a result of better nutrition of the population, allowing children the maximum opportunity of attaining their genotypic height and weight as early as possible.) The macroscopical development of the breast around puberty has been well illustrated by Tanner (1962). He divides it into five phases: first, the preadolescent elevation of the nipple, followed by (2) the beginning of mamillar growth and sprouting of subalveolar ductular breast tissue leading to the formation and protrusion of a small "breast hill" and an increase in the diameter of the areola. The next two stages are firstly a further growth of the nipple and secondly an accelerated growth of subareolar tissues. The fifth stage is the development of the shape of the mature breast.

The light-microscope appearance at the onset of puberty shows a development of both the glandular tissue and the surrounding stroma (Dawson 1934). Initially solid epithelial buds are formed from the

terminal part of the existing ducts, and these form branches. For the first time a surrounding loose connecting tissue around the ducts is described at this stage, and this stroma, as well as being looser than the rest of the supporting connective tissue, is also more cellular. The branching ductules are at this stage grouped into recognisable lobules. Dawson found that no corresponding glandular changes occur in the human male at puberty.

The hormonal changes at puberty begin with an alteration in the sensitivity of the negative feedback mechanism at the hypothalamus: in childhood the very low levels of circulating oestrogens are sufficient to prevent secretion of hypothalamic releasing factor (HRF) for follicle-stimulating hormone (FSH) and luteinising hormone (LH). At puberty the hypothalamic sensitivity to oestrogen is reduced, and secretion of HRF stimulates the production of FSH and LH, which in turn stimulate oestrogen production by the ovary (Short 1976). The stimulus which initiates this change is not known: the onset of menstruation has been correlated with body weight (Frisch and McArthur 1974), but other factors are thought to be involved.

The age at which hormonal changes begin is not known, and we do not know the precise endocrinological status (in terms of FSH and oestrogen levels) at the age of 11, when breast development first starts. However, it is known that ovulation does not occur before the menarche and indeed does not begin until several months after this event. According to Doring (1969), who investigated this question using basal body temperature record, 60% of menstrual cycles in the 12-14 age group are anovulatory, and 45% of cycles in the 15-17 age group are. Since progesterone is only produced by the corpus luteum after ovulation,

breast development will be well advanced before progesterone is first secreted,and indeed at the mean age of 15 breast development will be completed (in terms of gross morphology) without a substantial contribution from progesterone stimulation. Vorherr (1974) points out that progesterone is involved in the final stage of macroscopic maturation (Marshall's "fifth stage"), but it is doubtful if this contribution is essential.

Studies on other species have shown that cyclic gonadotrophin activity in the rat begins well before puberty (Baker and Kragt 1969) and that ovarian oestrogen production may cause rapid prepubertal growth of the mammary gland (Sinha and Tucker 1969a). A study of DNA, RNA and hydroxyproline content of the mammary gland in heifers (Sinha and Tucker 1969b) showed that mammary growth began at three months of age, which is three months before puberty in the breast studied. Animal studies show also that in some species mammary development is not "completed" at puberty, but proceeds during each oestrus cycle until pregnancy occurs. This has led to conjecture that a similar phenomenon may occur in the human: this is discussed in the next section.

Mean levels of circulating prolactin do not increase greatly at the time of puberty (Vorherr 1974), and it seems that the gross morphological development of the breast which is completed at puberty is the result of oestrogen stimulation (with a theoretical possibility of a contribution directly from pituitary gonadotrophins).

Although the development of the stromal component of the mammary gland is complete at the end of puberty, only partial development of the glandular component has occurred, compared with the full development

required for lactation. By far the greater part of the volume of the non-lactating breast is made up of fibrous tissue and fat. Morris (1977) has suggested that this difference in the development of the two components reflects the function of the non-lactating breast, which has no nutritive function (since pregnancy has not yet occurred) but functions to signal to the male that the female is sexually mature. Among other apes the female's receptivity is signalled by a swelling or colour change in the perineal area, and Morris suggests that when the human (the "naked ape") assumed the upright posture the function of sexual signalling moved from the perineal area to the mammary area. The hormone-dependent stroma of the breast - which appears to have nothing to do with the nutritive function of the glandular component - may therefore be directly analogous to the hormone-dependent perineal tissue of other species of ape. The probability that the stromal component has nothing to do with nutrition is discussed further below, during the discussion of pregnancy changes.

D Effects of the menstrual cycle on the breast

1 Light microscopy

Over the last fifty years various investigators have studied the lobules of the human mammary gland with a view to detecting cyclical changes, but there is no agreement on the influence of successive menstrual cycles on the detailed histology of the lobules and ducts. It is noteworthy that none of the investigations has looked separately at parous and nulliparous women.

The first worker to try to analyse the effects of the menstrual cycle

on the mammary gland was Rosenburg (1922), although earlier studies (Ott and Scott 1910; Ott and Scott 1914) had revealed that the corpus luteum could influence the development of the mammary gland in animals. Rosenburg performed post-mortem examinations on at least 56 women of reproductive age, in an attempt to correlate the appearance of the ovary with the histology of the breast. Most of the cases that he illustrates are nulliparae, although some had borne children, and he makes no distinction between the groups. It is of interest that much of his material came from the influenza epidemic of 1919: all the cases examined had died of infectious disease - some from tuberculosis. Rosenburg's conclusions were that the corpus luteum brings about a sprouting of the ductules of the breast, which explains the feeling of fullness that many women experience at the premenstrual phase. He concluded that the proliferation occurs in both parous women and nulliparae, and does not occur if the corpus luteum is absent. He described four phases - the pre-menstrual, menstrual, post-menstrual and interval phases - and illustrated spectacular proliferation during the first two, followed by involution (which he did not illustrate) progressing to a very sparse system of ducts in the interval phase. This description, he pointed out, differed from the standard anatomical teaching of his day: it is of interest that Rosenburg's 1922 description is the standard anatomical teaching of the present day.

In the same year McFarland (1922) published a study of the normal breast: he did not look for cyclical changes but was interested in the effects of pregnancy. He concluded that "there was no difference between the lobules of virgin breasts and those in the mammae of parous women".

Rosenburg's paper was followed by several reports in the German literature agreeing with his findings (Polano 1924; Ernst 1925; Litten 1926; Luchsinger Y Centeno 1927). Ernst (1925), confirming Rosenburg's findings, added that the involution after the menses resembled the involution after pregnancy (which is described in the next section). Litten (1926) agreed, and stated that secretion was present in the premenstrual phase.

However, three years after Rosenburg's report, Dieckmann (1925) re-analysed Rosenburg's material. The total number of cases described by Rosenburg was in fact only 25 (6 premenstrual, 9 menstrual and 5 each in the postmenstrual and interval phases). The average age of cases in the premenstrual phase was 29.8, in the menstrual phase 34.4, in the post-menstrual phase 24, and in the interval phase 22.6. Dieckmann illustrated cases of women dying after several months amenorrhoea, who had no corpus luteum on the ovary and yet who had lobules typical of Rosenburg's "menstrual" phase. Dieckmann's conclusions were that the corpus luteum is not essential for finding lobules in the breast, and that sprouting of the ductules is a function of age rather than the menstrual cycle. Menstrual changes such as the subjective feeling of fullness he attributed to oedema of the lobule. He did not, however, study the effects of parity, and again his subjects were post-mortem cases, the cause of death in most cases being chronic disease such as tuberculosis, nephritis, multiple sclerosis, etc.

Luchsinger Y Centeno (1927) agreed with Rosenburg rather than Dieckmann, but emphasised that the cycle did not have the same effect in all breasts, and said that cyclic changes were more pronounced in older women.

Ingleby (1932) also described cyclical changes, but although her description is very detailed it is not clear whether such changes had been observed from her own material. She found that tumours showed a similar cycle to normal breast tissue, and "it was found possible to diagnose the stage of the cycle from an examination of the tumour alone". This remarkable claim has never been confirmed, and the same author ten years later (Ingleby 1942) had modified her original enthusiasm. Far from claiming, as she had in 1932, that "premenstrual proliferation is much more rapid than carcinomatous growth", she remarked (after examining whole breast sections) that "present knowledge of the sexual cycle is fragmentary. Without a large number of normal specimens - which I have been unable to obtain - it would be absurd to dogmatise." While emphasising the extensive variation in a single breast, Ingleby (1942) still felt that enormous growth and regression can take place - but "only some lobules undergo changes in a cycle, and they may not do so simultaneously" (Ingleby and Gershon-Cohen 1954).

In 1934, EK Dawson of Edinburgh published her classic study of the normal breast. She found that growth of the lobules begins at puberty and continues until general bodily maturity is reached. According to Dawson, "changes associated with the recurring sexual cycle are superimposed on the general growth initiated with the approach of puberty, but the actual changes in human mammary tissue during the cycle have yet to be defined. Such definition would appear to demand a careful estimate of data which indicate sexual and general maturity, as well as knowledge of the menstrual phase, and in the absence of these details, comparison of tissue from different individuals may be misleading." Dawson was therefore reluctant to make categorical

statements about the effect of the cycle on the breast, but she does record that "lobules are always present during the menstrual interval in tissue which is not yet approaching post-menopausal involution", contradicting Rosenburg's suggestion that lobules are re-formed each month. Dawson illustrated lobules at different phases of the cycle with little difference in glandular content, but she felt that a typical premenstrual lobule had loose lobular connective tissue ("physiological oedema") which disappeared in the intermenstrual phase.

At the same time Lewis and Geschickter (1934) published a study of 44 normal breast biopsies taken at operation for carcinoma or adenoma. They described the following changes: at mid-cycle there is an expansion of the duct system and an increase in the epithelial thickness. Just before the menses there is an increase in acinar elements. At the time of the menses there is a round cell infiltrate in the stroma, and desquamation of the duct epithelium. The post-menstrual phase is characterised by disappearance of this infiltrate, a decrease in the size of the acini, and the stroma's becoming more compact.

Taylor (1936) reviewed 41 biopsies of normal tissue, and pointed out that there is much variability between breasts. "The chief feature of this series was the immediately evident variability of the lobular development of different breasts, irrespective of the time of the cycle. This is in direct accord with Dieckmann who recognised a mature and an immature type of lobule somewhat dependent on the patient's age. This variability makes difficult attempts to prove the occurrence of premenstrual proliferation of the ducts by comparing single sections from different individuals, but makes it readily possible to arrange sections to

illustrate any special morphological process that one may expect to find. Taking Figs 1 and 2 one has the evidence to demonstrate the Rosenburg theory, but the reverse can as readily be shown using Figs 3 and 4." He was able to take consecutive biopsies at day 14 and day 28 from one patient (who was, however, suffering from marked painful hypertrophy of the breasts) and he found no change. In general, he felt that acini became slightly dilated before menstruation, and the cells became more distinct in outline. He also felt that the intralobular connective tissue fibres were widely separated before menstruation, and that there was "obvious" premenstrual hyperaemia, but these were the only changes seen.

Grynfeltt (1938) agreed that proliferative changes occurred in the premenstrual phase, though his main interest was centred on the appearance of secretion in the breast, and he appears to have accepted the idea of premenstrual proliferation uncritically. He examined tissue only from nulliparae (the source of the tissue is unclear, as is the number of subjects examined), and concluded that a secretion appears in the epithelial cells during the premenstruum, apparently similar to colostrum. He pointed out that there is no indication in the literature as to when in pregnancy functional changes start appearing in the breast, and contended that secretory activity begins during the luteal phase.

In a large study of 300 cancer-containing breasts and 200 specimens of noncancerous breast tissue, Foote and Stewart (1945) attempted to find structural differences between the breast of women who have breast cancer and those who do not have the disease. In their description of the mammary lobules, they remark that "examination of the character

of the mammary lobules has offered more difficulties than any other aspect of this morphological analysis". They found it impossible to find a survey of normal material taken from patients without chronic disease, but after studying a large amount of material they concluded that "certain lobule patterns are more characteristic of one phase of the cycle than another". Their findings were, they reported, more or less in agreement with those of Rosenburg and Dieckmann, but they emphasised the considerable heterogeneity of appearances within a single breast. The characteristic lobule patterns that they describe are as follows.

On day 0 of the cycle, acini are numerous and closely spaced, and lumina are open and contain secretion. Lining cells are orderly, cuboidal to columnar, and the cytoplasm is rather abundant and pale. On day 4 there are still numerous acini, but some lumina are narrowed and many are closed. There are fewer columnar cells, and contracture of acini leads to obliteration of lumina, and cells piling up in a disorderly fashion. The stroma shows early condensation, decreased vascularity and increased numbers of lymphocytes. On day 8 there are still more lymphocytes, collapsed acini, smaller acinar cells with nuclear pyknosis and a dense stroma. By day 12 involution is nearly complete: there are few acini, collapsed with hyaline connective tissue condensation around them. The lymphocytic infiltration has cleared. Reactivation occurs at 15 days: the stroma loosens, the connective tissue cells become larger and acinar lumina are re-established. By day 22 the acini have multiplied, the lining cells are larger and taller, with more abundant cytoplasm and pale nuclei. There are occasional mitoses, and the lumina are present but not yet crowded with secretion.

Foote and Stewart are clearly unhappy about the heterogeneity of the lobules in their material, and record that it was possible to predict the phase of the menstrual cycle correctly in only 10% of these cases. They suggest that this might be due to the primary condition requiring surgery, and so they examined the breasts of 27 women who were victims of sudden death, to see if "normal" breasts showed a similar heterogeneity. They report that the heterogeneity among this group was much less: 22 of the subjects were suitable for analysis (the remainder being post-menopausal or having advanced post-mortem changes) and of these 22 the phase of the menstrual cycle was correctly predicted in 16 (72%). Details of this prediction (eg. how many phases the cycle was divided into) are not given, but the prediction was apparently made by two independent observers working "blind".

Engel (1947) studied 100μ sections of whole human breast and commented on "alveolar sprouting in the premenstruum", which he said only occurred in breasts which already consisted of well-differentiated glands. He pointed out that neither size nor palpation gives any correct indication of the functional capacity of a breast. Asking the question, "Do menstrual changes occur in the breast?" he replied, "Because of the natural variations in the glandular equipment of different breasts it can be said that there is no rule and menstrual changes may or may not occur".

Dabelow (1957), summarising the work of various investigators, including his own earlier work, also draws attention to the fact that changes in a gland are not uniform and that some parts of the gland are not affected at all. He describes partial lobular degeneration

after the menses, of varying intensity, and states that cellular infiltration occurs during degeneration and regeneration. According to Dabelow, all authors agree that stromal oedema, lobular enlargement and partial new formation occur, and he states further that over a period of years each cycle adds more differentiation and some lobuli, since not all lobuli degenerate. Later, after describing involution, Dabelow states that "the degree of involution never quite resembles that state of the virgin breast", but he does not explain how the virgin and the post-involution breasts differ.

Huseby and Thomas (1954) studied the effect of exogenous oestrogen (given for breast cancer) in 36 patients all of whom were more than five years past the menopause. 34 of them showed stimulation of the epithelial cells, which were enlarged and showed signs of increased activity. There was proliferation of ductules and new lobule formation, and an increase in interlobular connective tissue. The oestrogen did not, however, restore breast histology to a normal pre-menopausal appearance.

Ozzello and Speer (1958) studied normal breast tissue obtained from 42 patients (20 from patients with carcinoma, 17 with fibrocystic disease, and 5 with fibroadenomata). These investigators were particularly interested in the stroma of the breast, which they studied with special stains (haematoxylin and eosin; the Ritter-Oleson method for neutral polysaccharides; periodic acid-Schiff stain for neutral polysaccharides; and toluidine blue). They found that the fibres forming the basement membrane formed a tight network in close contact with the myoepithelial cells. The stroma surrounding the basement membrane was RO positive, but the interlobular stroma was predominantly PAS positive. The interlobular stroma did not change its reaction to

any of the stains during the cycle, but the intralobular stroma showed characteristic cyclic alterations. During the intermenstrual phase it was in part PAS and in part HS positive, the former predominating somewhat over the latter. The premenstrual phase was characterised by an increase in the HS positivity, and increased metachromasia: this increase was concomitant with an increase in the looseness of the stroma and in the number of fibroblasts. The PAS positive material disappeared, and at the end of the premenstrual phase PAS positive secretory granules appeared within the acinar cells and PAS positive material appeared in the lumina of the ductules. After menstruation these changes were reversed.

CD Haagensen, in his textbook on diseases of the breast (Haagensen 1971), noted that there was no consensus on histological changes that are supposed to occur during the cycle. In an effort to settle the controversy he carried out a histological study of surgically removed breast tissue, counting the numbers of lobules and acini in ten representative microscopic fields of each of 100 specimens. (The diagnosis of the primary condition is not recorded). He found no difference in the menstrual, premenstrual and interval phases. Nor could he see any variation in numbers of mitoses, compactness of acini, cellularity of the stroma or intralobular oedema. The only change he found was that vacuolisation of the basal layer of cells was present in 33% of cases in the premenstrual phase and 12% of cases in the interval phase: its significance, he felt, was questionable. Unfortunately, the parity of the subjects examined is not recorded. Remarking that the change in size of breasts in the premenstrual phase (well documented by several investigators, and discussed in Chapter 3) must have some basis, he suggests that it must be due to blood or lymph engorgement,

or to increase in the extracellular fluid tension - changes not demonstrable by conventional microscopic techniques.

Nizze (1972) studied the intralobular connective tissue in 99 healthy breasts obtained at autopsy and 54 at biopsy. He noted a loosening of the fibres, which he attributed to oestrogen and progesterone - the oedema of the connective tissue was progesterone-induced with a permissive effect from oestrogen, but oestrogen alone produced increased density of connective tissue. These conclusions were inferred from the varying ages of the subjects, rather than from plasma hormone assays.

Other methods of investigating cyclical changes in the breast are worth mentioning here. Pickles (1950) developed a baroplethysmographic method of measuring blood flow in the breast, and made measurements on one subject during the normal cycle as well as during pregnancy and lactation (Pickles 1953). He found that blood flow increased during the luteal phase to a maximum just before menstruation, and then fell to its lowest point at about the 10th day of the cycle. The change was of the order of 40% of the units he used, but his method did not allow calculations to be made of the amount of blood flowing. Papanicolau (1958) in a study of exfoliative cytology of nipple secretion found that among women aged 20-39 secretion could be aspirated from 23.8% in the second week of the cycle and from 55% in the fourth week: details of parity are not given, but the cyclical change was not seen in women over forty. Petrakis (1975) stated that secretions could be aspirated from approximately 75% of non-lactating premenopausal women. In a study of 606 women in San Francisco, he found that parity did not affect the number of women from whom

secretions could be obtained (68.9% of nulliparous women and 71.6% of parous women) and the menstrual cycle did not affect the proportions either (70.8% in week 1; 83.9% in week 2; 75.8% in week 3; and 82.8% in week 4). However, the proportion fell to 60% after the menopause.

2 Ultrastructure

The ultrastructure of the normal human breast was first studied in the late 1950s, and none of the early reports on normal tissue suggested cyclical changes (Tannenbaum et al 1969; Ozzello 1971) although these were looked for carefully in 18 patients by Waugh and Van der Hoeven (1962), who also found no effect of parity. In a detailed electron microscope study of the epithelial-stromal junction Ozzello (1970) suggested that it might play a functional role in regulating the transfer of substances from the bloodstream to the mammary epithelium and from the latter to the lymphatics, possibly by varying the concentration or the chemical configuration of its acid mucopoly-saccharides. However, there was no evidence of a change in its structure during the cycle.

A study in 1974 of 21 women of reproductive age (Fanger and Ree, 1974) suggested that ultrastructural changes do occur in the epithelial cells. Nine of the patients examined were parous, but parous and nulliparous women were not analysed separately. All their subjects had benign breast disease, and the tissue studied was histologically normal. Fanger and Ree found "characteristic" light microscopic changes between the follicular and luteal phases of the cycle - in the preovulatory phase acini were numerous, closely-spaced and had nearly obliterated lumina. Epithelial cells were small, crowded together and had irregular dark nuclei: their cytoplasm was pale and "vacuolar fixation artefact" more frequent. The stroma was

compact with common lymphocytes. In the postovulatory phase the acini were larger, with re-established lumina. Epithelial cells were taller, less crowded and had paler larger nuclei. Rare mitoses were seen, cytoplasm was abundant, and the stroma was oedematous. "Lobulo-acinar organisation" was at its maximum.

Under the electron microscope Fanger and Ree were able to divide the cycle into two phases, I and II, and subdivide each phase into two, according to the appearance of the epithelial cells lining the ductules (which were the only structures examined in this study). A phase I cell (soon after the menses) had a moderately wavy plasmalemmal outline with occasional widening of intercellular spaces: microvilli were not prominent, and nuclei varied from pear shaped to irregular. The cytoplasmic appearances - ribosomes, polysomes, rough endoplasmic reticulum and golgi complexes - were poorly developed, suggesting relative inactivity. The Phase IA cell was swollen with straight plasmalemmal outlines obliterating intercellular spaces: microvilli were at their smallest. The nuclei were swollen, but otherwise the cells seemed inactive - the difference between I and IA being the swelling of the IA cells, possibly due to increased intracellular fluid content. Phase IIA follows: the plasmalemmal outline is very irregular, as is the nucleus. The cytoplasm is denser, with dispersed glycogen particles. Phase IIA starts at day 15, but there is still little evidence of activity in the cytoplasm, apart from the increased density. Phase II cells, by contrast, have a slightly irregular outline, with microvilli at their most prominent and numerous. The nuclei are enlarged and less irregular, and the nucleoplasm is moderately dense, suggesting increased activity. The density of the cytoplasm is variable, with the glycogen leached out, but electron-opaque material

present. The rough endoplasmic reticulum, ribosomes, polysomes and golgi apparatus are enlarged and suggest potential secretion.

Fanger and Ree state that all the cells in a particular specimen do not show the same type, but the predominant type in most cases corresponds to the stage outlined above. They suggest that the phase I cells, apparently inactive, correspond to the low ovarian hormone levels of the menstrual phase, and the phase IA cells, which are swollen, correspond with raised oestrogen levels in the follicular phase - oestrogen influences cellular water and electrolyte metabolism. They suggest also that their phase IA cells may correspond to Bassler's B cells (Bassler 1970), and that the appearance of progesterone in the blood stimulates glycogen production (progesterone causes glycogen production in endometrial cells). However, they did not confirm that their patients were ovulating. The changes in glycogen content of endometrial cells during the luteal phase of the cycle have been described by Armstrong (1973): glycogen and mitochondria appear between the 13th and 20th days and an accumulation of glycogen occurs in the cell from the 23rd to 28th day: the nucleus of the endometrial cell is smooth in the proliferative phase and irregular in the secretory phase.

The changes described by Fanger and Ree are the appearances which would be expected, but it should be noted that they give no figures on the frequency of their "typical" cells in any particular specimen, and so their observations should be treated with some caution.

E Pregnancy

The macroscopic changes in the breast in pregnancy are an enlargement of the breast, with the blood vessels becoming more obvious, an increase in size and pigmentation of the areola, and a change in consistency to

a firmer and slightly less regular organ. Subjectively, "breast tenderness" is one of the first signs of pregnancy, and is said to begin during the fifth to sixth weeks after the last menstrual period.

The volume change during pregnancy has been fully studied by Hytten (1954). Using a water-displacement technique which involved a specially-made bell-shaped apparatus, he measured breast volume during pregnancy in 86 subjects, and then measured the subsequent milk yield. He found that the main increase in volume occurred in the middle trimester of pregnancy (though the increase during the very first weeks, before the women came under his care, is not known). He also found that the subsequent milk yield does not correlate with the total breast volume, but correlates much more closely with the increase in volume during the pregnancy. This finding emphasises the point which was made earlier, that the existence of a stroma, increasing the volume of the breast, is not important to its nutritive function, since the nutritive role of the breast seems to be best served by an increase in size caused by the proliferating glandular component during pregnancy.

The histological changes in pregnancy are said to begin within the first 3-4 weeks gestation, with an increased ductular sprouting. The proliferation of the ducts continues until halfway through pregnancy (Vorherr 1974) and is then gradually replaced by a proliferation of lobules and a hypertrophy of existing lobulo-alveolar structures. During the third trimester of pregnancy the breast consists mainly of enlarged lobules, with only a little supporting stroma between them. Dabelow (1957) states that the amount of connective tissue is relatively decreased, but Dawson (1935) states that there is an actual decrease in the amount of connective tissue, though she points out that there is

much variation between individuals. The development during the latter stages of pregnancy consists of differentiation and maturation of secretory cells: the alveoli lose their two-layered structure during the second half of pregnancy, though the milk ducts retain a two-layered epithelium throughout pregnancy and lactation. As the end of the pregnancy approaches, fat droplets are seen within the secretory cells, and there is an increasing accumulation of colostrum within the lumina of the alveoli. The increase of breast size towards the end of pregnancy is thought to be due to an increase in the dilation of alveolar lumina by secretion (Vorherr 1974): this would fit in with Hytten's (1954) finding that the increase in breast volume during pregnancy is correlated with subsequent milk yield.

During pregnancy the levels of circulating oestrogen, progesterone and prolactin (Friesen 1972) increase steadily to achieve maximum values near term. Chorionic gonadotrophin reaches its maximum value at the tenth week of pregnancy and then declines to a steady level after the sixteenth week. The histological development of the breast, which continues throughout pregnancy, thus correlates more closely with the steady increase in steroid levels than with the early peak of chorionic gonadotrophin. Human placental lactogen, however, also shows a steady increase during pregnancy, and the pituitary gland undergoes changes in pregnancy which involve a 30-50% increase in its weight and volume (Vorherr 1974), mainly due to "prolactin cell" hyperplasia (Goluboff and Ezrin 1969). The secretion of pituitary gonadotrophins and growth hormone decreases during pregnancy (Jaffe et al 1969; Hanson et al 1970), but serum corticosteroid levels are increased in late pregnancy (Scholz and Huther 1971).

Work on hypophysectomised animals showed that the pituitary synergises with oestrogen and progesterone in stimulating mammary development (Turner 1939). Later work suggested that prolactin alone could produce full mammary development in rats (Meites 1965). However, the DNA content of the gland in hypophysectomised rats is lower than in ovariectomised rats (Hahn and Turner 1966). Mammary development can be achieved by injection of oestrogen and progesterone (Moon et al 1959) but more complete development requires growth hormone, insulin and cortisol (Kumaresan 1967).

It seems, therefore, that most hormones can be implicated to some extent in the mammary proliferation of pregnancy, but the exact contribution of each remains unclear. The classical view that oestrogens stimulate duct growth while progesterone produces lobulo-alveolar development was in vogue before the importance of prolactin or human placental lactogen was realised. The latter hormone is very similar in structure to human growth hormone (Sherwood et al 1971) and its concentration steadily increases during pregnancy (Varma et al 1971). Its function is unclear, as the lactogenic activity that it shows in some experimental situations must be inhibited during pregnancy, when it is produced: whether it also has a role in stimulating mammary development is not known.

F Lactation

A full account of milk production will not be given here: the mechanisms of secretion of fat, protein, carbohydrate, ions and water have been reviewed by Linzell and Peaker (1971). To summarise the relevant points in the human: colostrum is produced during the first

3-5 days after parturition, before the milk "comes in". Colostrum is a cloudy yellowish fluid with a specific gravity slightly higher than that of milk. It is rich in protein and in some ions such as sodium and potassium, and is particularly rich in immunoglobulin, especially IgA. The importance of this to the neonate, particularly in respect of the passive transfer of immunity, has been appreciated only relatively recently, and is discussed in Chapter 6.

Around day 2-5 postpartum, there is a transitional phase, during which the concentration of protein in the secretion falls and that of lactose and fat rises. The secretion of fat droplets into milk involves the pinching off of part of the apical membrane of the secretory cell, but whole cells are not lost during milk production. During lactation, therefore, the histological structure of the breast remains static, with the alveolar cells having the appearance of actively secreting epithelium: they have microvilli on the luminal surface, a large Golgi apparatus near the basal nucleus, and prominent mitochondria and rough-surfaced endoplasmic reticulum. The lumina of the alveoli are distended with secretion: storage of milk is effected in the human mainly by the alveoli, with very little contribution from the lactiferous sinuses near the nipple. Engel (1947) studying whole-breast sections, noted that the lactating gland has alveoli lined by a single layer of cells (unlike the two-layered arrangement in the non-lactating breast). He felt that the outer layer of cells was the precursor layer, in the non-lactating breast, and the inner layer "a kind of protective covering".

The alveoli are surrounded by a basket-shaped network of myoepithelial cells, which are the effector organs in the suckling reflex. The

suckling reflex is initiated by stimulation of the nipple and areola, though conditioning allows milk ejection to be produced purely by psychological stimuli such as the sound of the infant's crying. The afferent component of the reflex stimulates oxytocin release from the posterior pituitary by a neuronal pathway involving the supraoptic and paraventricular nuclei of the hypothalamus. The oxytocin acts on the myoepithelial cells to stimulate contraction: no innervation of these cells has been demonstrated, and they are said to be 10-20 times more sensitive to oxytocin than myometrial cells (Vorherr 1974). The structure of the myoepithelial cells under the light microscope has been described by Richardson (1950): they are very elongated cells with branching processes, and lie external to the basement membrane. They are irregular in shape and orientation, and are usually regarded as modified epithelial cells. Controversy about the exact role of myoepithelia continues, but doubts about their ability to function as contractile tissues were ended with the advent of electron microscopy, which demonstrated the presence of myofibrils (Hamperl 1970; Hollman 1974).

The endocrine control of lactation has two aspects: induction of lactation and its maintenance. Induction - the sudden appearance of milk within a few days of parturition - is thought to be the result of the interaction of several hormones. Prolactin is essential, and hypophysectomy during pregnancy means that lactation will not occur. However, highly purified prolactin is ineffective in initiating lactation in experimental animals unless combined with adrenal glucocorticoids. Oestrogens can produce lactation also, but this is thought to be mediated via prolactin (Tindal and Knaggs 1966). In humans the secretion of oestrogens, progesterone and prolactin is

increased in late pregnancy and the levels of steroid secretion fall after parturition. It is thought that progesterone has an inhibitory effect on the secretory tissue, and that the initiation of lactation is caused partly by the removal of this progesterone effect (Turkington and Hill 1969; Kuhn 1969; Herrenkohl 1971). Progesterone is also said to inhibit the stimulatory effects of oestrogen and prolactin secretion in rats (Chen and Meites 1970). In vitro induction of lactation requires a specific sequence of hormones: insulin and hydrocortisone are used first to induce mammary cell division, and following this prolactin in combination with insulin and hydrocortisone will induce casein production (Topper 1970). Stimulation of α-lactalbumin synthesis can be blocked by progesterone (Turkington and Hill 1969).

For the maintenance of lactation, both suckling and adequate hormone levels are required, though suckling is not necessary for the initiation of milk synthesis. Again, a number of hormones have been shown to affect the maintenance of lactation (fully reviewed by Cowie et al 1980): in animal experiments hypophysectomy causes cessation of milk synthesis (Selye 1933) and hormone replacement with prolactin and ACTH will partially restore lactation in hypophysectomised rats (Cowie 1957). In goats lactation can be completely restored using a combination of prolactin, growth hormone, adrenal corticosteroids, and tri-iodothyronine (Cowie 1969a). Prolactin alone will restore lactation in rabbits (Cowie et al 1969). Suckling causes prolactin secretion (Tucker 1971) but the levels steadily decline as lactation progresses (Kaprowski and Tucker 1973a). Animal studies have also shown the importance of ACTH and adrenal steroids, of the thyroid and parathyroid, and of insulin (Tucker 1974): since these hormones are essential to

life, it is not surprising that their removal will affect lactation.

Oestrogens are not essential to lactation: the circulating levels of ovarian oestrogens are low during lactation, and ovariectomy in various animal species does not affect lactation (Folley and Kon 1938; Griffith and Turner 1962). Oestrogens have in fact an inhibitory effect on lactation in several species (Cowie 1961), and synthetic oestrogens have been used to suppress lactation in women (Watson 1969). Progesterone does not affect lactation, even if large doses are given (Folley 1942), but will enhance the suppression of lactation produced by oestrogen (Griffith and Turner 1962). Progesterone may interfere with the milk ejection reflex: Griffith and Turner found that exogenous oxytocin did not empty the gland after progesterone administration - an observation which suggests an action of progesterone on the myo-epithelial cells themselves.

Although ovarian steroids do not have important physiological effects on lactation, lactation affects their production. In the human, lactation produces a state of amenorrhoea which lasts for a variable length of time but is usually several months. It has been suggested that the inhibition of ovulation which is produced by lactation has been the main factor regulating population growth until relatively recently in human history (Short 1976). The inhibition of ovarian activity appears to be achieved at a hypothalamic level by neural stimuli: denervation of the mammary glands of animals prevents the inhibition of reproductive activity as well as preventing the surge of prolactin induced by suckling (Kann and Martinet 1975). If the afferent neural stimuli do act at a hypothalamic level, then the secretion of releasing factors for the gonadotrophins could also be

inhibited: on the other hand it has been shown that prolactin itself can inhibit ovarian activity in vitro (McNatty, Sawers and McNeilly 1974), and the suckling-induced surges of prolactin may therefore act directly on the ovary.

G Involution

If suckling is discontinued for more than 48 hours, the rate of milk synthesis and milk secretion rapidly begins to decrease (Dabelow 1957). Bonnar et al (1975) showed that plasma prolactin concentration falls to normal within three weeks of delivery if breast feeding is discontinued, but remains raised for six weeks if breast feeding is proceeded with. In both groups of women oestrogen levels fall at delivery and FSH rises, but in non-breast feeders oestrogen rises after 17 days in response to FSH. Bonnar suggests that prolactin inhibits ovarian response to FSH. Engel (1947), measuring milk output by test-feeding, stated that menstruation only returns after lactation has begun to fail.

Dawson (1935) in her histological study of the breast describes six stages in involution, beginning with distension of the alveoli with retained secretion, which may actually rupture the alveolar wall. There is flattening of the epithelial cells, and then phagocytic cells migrate into the alveolar lumen. Secretion is removed, distension diminished, and the alveolar wall desquamates:the cells are pushed into the lumen, and gradually disintegrate. The process is irregular and does not proceed uniformly even within the same lobule, but Dawson felt that the alveoli at the periphery of a lobule seemed to disintegrate first. The next stage is one of fibrosis, with the re-growth of fibrous tissue, and the last stage is the reappearance of fatty tissue.

Vorherr (1974) describes an essentially similar process, with the addition that intracellular lysosomal enzymes are induced near the Golgi apparatus to remove intracellular secretory material.

The duration of involution obviously depends on whether lactation is stopped suddenly or gradually: Vorherr (1974) quotes a time of three months, and Dawson 9-12 months. According to Holmann (1974), the myoepithelial cells are the structures least affected by the disorganisation of involution.

H The gland after the menopause

At the time of the menopause, which occurs around the age of 50, ovarian activity ceases and circulating levels of oestrogen and progesterone fall. There is a reduction of the amount of glandular tissue in the breast: according to Vorherr (1974) this begins prior to the menopause, around the age of 35, and the process speeds up after the age of 45. Loss of lobular and alveolar structures occurs, and ultimately only fat, connective tissue and the mammary ducts remain. Reduction in the amount of stroma is said not to occur. Thus the menopause does not produce a dramatic change in breast structure: the loss of glandular tissue is a long-term process continuing over the decades after the age of 45. This tends to contradict those who believe that the ovary exerts an immediate effect on breast histology.

SUMMARY

This brief outline of the appearances of the human mammary gland during a woman's lifetime indicates that although lactation and its endocrine control have been well investigated, there remains uncertainty

about the effects of endogenous hormones on the non-pregnant and non-lactating gland. Some investigations have shown cyclical changes in the breast lobules during the normal menstrual cycle, but these changes have not been quantified and have not been related to measurement of hormone concentrations. Other investigators have found no cyclical changes, while still others have compromised by suggesting that in any one cycle changes occur in some lobules but not in others. None of these previous studies has investigated cyclical changes separately in nulliparous and parous women.

Before the present study of breast histology is described in Chapter 4, Chapter 3 will present the results of a study of breast volume changes during the menstrual cycle.

Chapter 3

VOLUME CHANGES

I INTRODUCTION

Many women report a feeling of fullness in the breasts during the premenstrual phase of the cycle. There has, however, been only one previous attempt to measure breast volume changes directly (Ingleby, 1949). No previous investigation had addressed the question of whether or not oral contraception produces similar changes in breast volume. In the previous investigation by Ingleby, the parity of her subjects was not recorded. It was therefore thought worthwhile to repeat the direct measurement of breast volume changes, and also to measure breast volume in women taking the oral contraceptive pill.

This study was conducted by a student in the Honours Zoology course at Edinburgh University, Miss Dorothy Milligan. She was under the supervision of Professor Short and myself

II METHODS

A Subjects

Four women were studied, all of them nulliparous healthy women aged 21 years.

Subject 1: Measurements were made for one normal cycle followed by one contraceptive-controlled cycle. The oral contraceptive was Norinyl 1 (mestranol 50µg and norethisterone 1 mg), taken for 21 days followed by seven tablet-free days. This subject had taken Norinyl 1 before the study and measurements were begun during the second normal cycle after stopping the contraceptive.

Subject 2: Measurements were made for one contraceptive-controlled cycle, followed by a normal cycle and then another contraceptive-controlled cycle. The oral contraceptive used was Ortho-Novin 1/50 (mestranol 50µg and norethisterone 1 mg), and measurements were begun on the sixth day of treatment. Ovulation was confirmed during the normal cycle, by serial progesterone determinations.

Subject 3: Measurements were made for three consecutive contraceptive-controlled cycles. The contraceptive used was Gynovlar 21 (ethinyl-oestradiol 50 µg and norethisterone acetate 3 mg) taken in the usual regime of 21 days treatment followed by seven tablet-free days.

Subject 4: Measurements were made for one normal 29-day cycle. This subject had not taken oral contraceptives.

B Method

1 Measurement technique A glass mixing bowl 7 inches in diameter (17.8 cm) standing inside a container on the floor was filled to the brim with water. The woman, kneeling on the floor, lowered one breast into the bowl, displacing the water into the surrounding container. The volume of water displaced was measured in a 1-litre graduated cylinder. Variability due to postural changes was controlled by marking positions for the container, hands, knees and elbows on a sheet of plastic. Each woman made three consecutive measurements on each breast every day at the same time, using water of about the same temperature. Repeated measurements were also taken from one woman throughout one day, and the results related to previous posture. A series of consecutive measurements was also made over 40 minutes on the right breast of one woman at water temperature ranging from 45 - 15^{o}C.

2 Reliability of technique: A measure of the precision of the technique was obtained from the correlation between measurements made on left and right breasts for each day of the cycle. The correlation coefficient was highly significant ($P<0.001$) for all individual cycles (Table 3:1). The "error" in the method was calculated by expressing the variation between consecutive measurements made on one breast on any one day as a percentage of the total change in volume during the cycle. Table 3:1 shows the error for each subject expressed as a coefficient of variation.

TABLE 3:1

Correlation between measurements on left and right breasts, showing precision of technique

Subject	Correlation coefficient between measurements on left and right breasts	Overall coefficient of variation
1	0.859	2.1
2	0.728	8.5
3	0.865	3.2
4	0.889	5.4

III RESULTS

A Effect of previous posture

Posture had an effect on breast volume, as shown in Table 3:2 below. This effect was significant ($P<0.001$).

TABLE 3:2

Variations in breast volume after consecutive changes in posture

	Horizontal for		Vertical for	
	4 hours	11.5 hours	4 hours	11.5 hours
Right breast (ml)	523	552	532	562
Left breast (ml)	530	588	543	600

B Effect of water temperature

There was a decrease in volume with decreasing water temperature and this decrease was significant ($0.05>P>0.02$). The figures are given below in Table 3:3.

TABLE 3:3

Variation in breast volume with consecutive decreases in water temperature

Temperature (^{o}C)	45	40	35	30	25	20	15
Breast volume (ml)	542	564	571	514	525	505	518

C Cyclical changes

Breast volume increased significantly during the second half of both normal and contraceptive-controlled menstrual cycles. Fig 3:1 shows consecutive cycles in a single subject, the first being normal and the second contraceptive-controlled. The difference between the mean volumes in the first and second halves of both cycles is significant ($P<0.001$). In the cycles illustrated in Fig 1 the smallest volumes are found on days 9-17 of the normal cycle, with a steep rise until the maximum volume is reached on day 25. The difference between the minimum and maximum volumes is 100 ml - ie. 20% of the minimum volume.

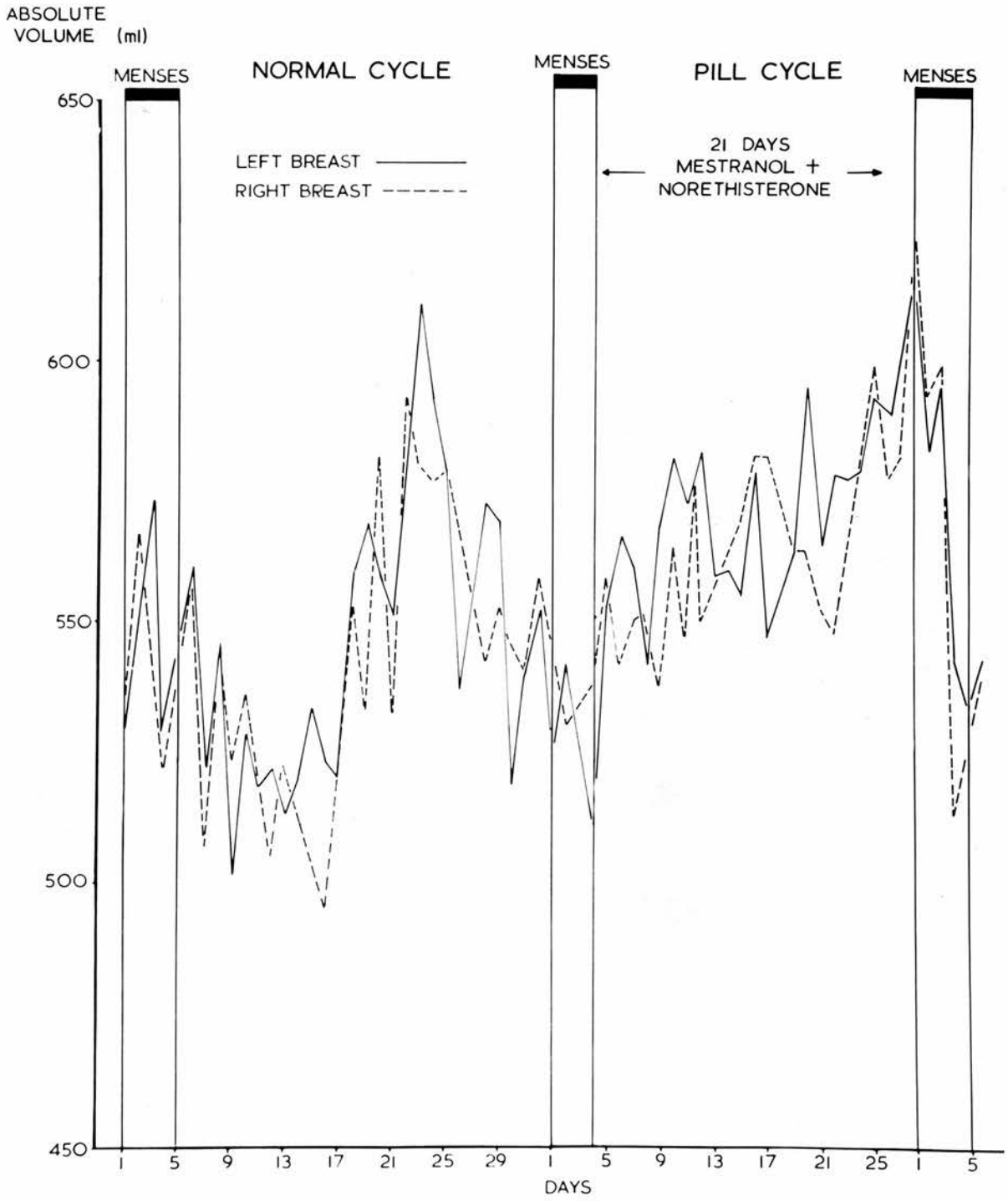


Fig 3:1 Subject 2: absolute volume changes with time throughout a normal cycle and the subsequent contraceptive controlled cycle. (From Milligan, Drife and Short, 1975).

In the contraceptive-controlled cycle in Fig 1, the minimum volumes are found during menstruation, with a steady increase during the 21-day course of tablets until the withdrawal bleed, when there was a sharp fall.

Fig 3:2 shows the volume changes in all three normal cycles studied. Although the peak volumes occur in the second half of the cycle, their relationship to menstruation is not consistent, with two of the subjects showing peak volumes at the start of the menses, and the third showing the peak volume during the luteal phase.

Fig 3:3 shows all six contraceptive-controlled cycles studied. Although the decrease in breast volume in these cycles started on different days in different women, in every case the main decrease occurred during the week when the pill was not taken. Breast volumes increased again with the start of a new course of treatment, and maximal volumes were found at the time of withdrawal bleeding in two subjects, and during the latter part of the treatment cycle in the three cycles from the third subject. Minimum volumes appear to occur a week earlier in contraceptive-controlled cycles than in normal menstrual cycles.

IV DISCUSSION

The first investigators to measure morphological variations in the breasts during the menstrual cycle were Reiman and Seabold (1933). They took a series of X-rays of the breast and measured the area of the breast shadow using a planimeter. They found that the breasts were largest 7-10 days before the menses, although they noted that there was a variation between different individuals, between different months with one individual, and even between different parts of the

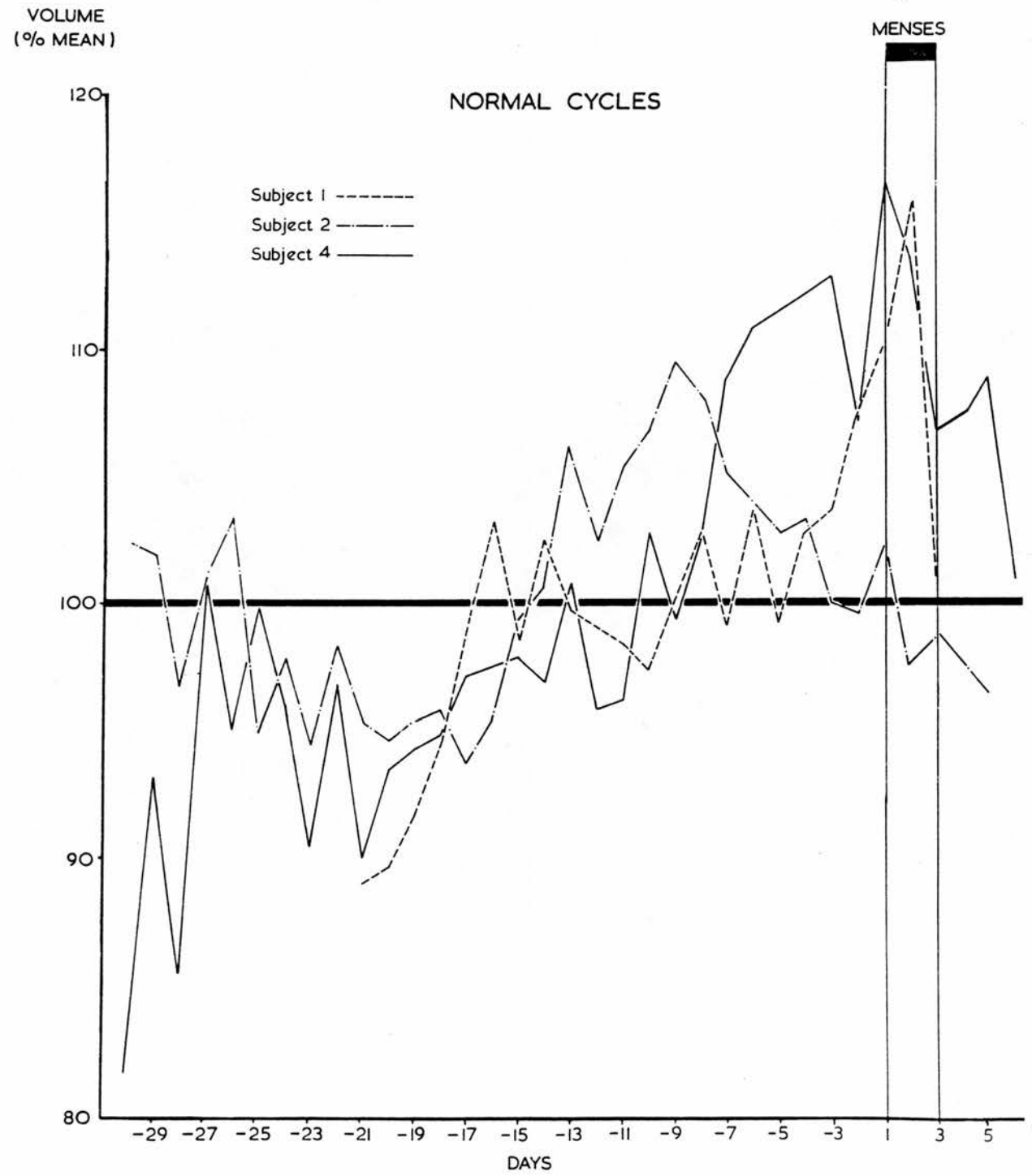


Fig 3:2 Volume changes throughout all complete normal cycles expressed as percentages of mean volume for each cycle, and plotted backwards from the first day of the menses. (From Milligan, Drife and Short, 1975).

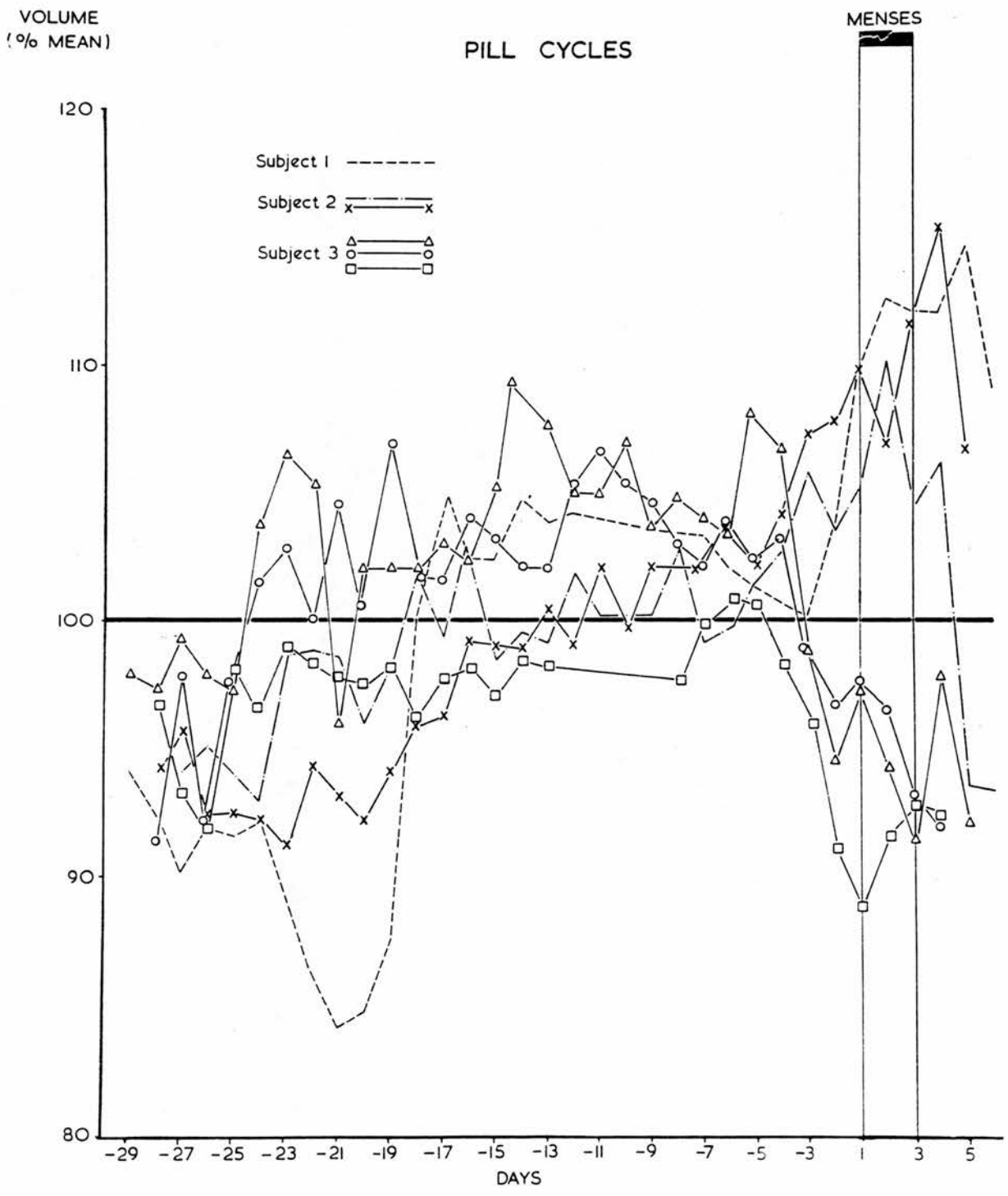


Fig 3:3 Volume changes throughout all complete contraceptive-controlled cycles expressed as percentages of mean volume for each cycle, and plotted backwards from first day of menses. (From Milligan, Drife and Short, 1975).

same breast. The variation in size found by these workers was up to 40% of the smallest volume, and they also apparently found that a benign tumour varied in size with the cycle. This type of investigation has of course never been repeated because the dangers of repeated X-rays are now recognised. Reiman and Seabold felt that their investigation gave an indication of volume change only, and that accurate measurement of breast volume was impossible.

However, Geschickter (1945) used a water displacement technique and observed an increase in volume in the second half of the cycle. Ingleby (1949) made weekly plaster casts of the breasts of volunteers, and then weighed the wax impressions made from these casts. She found the smallest breast volumes in the follicular phase of the cycle in seven out of nine women, with an increase of 8-44% above minimum values during the second half of the cycle.

Although a simple one, the technique of measurement used here seems a reliable one, as judged by the high correlation coefficients obtained by each woman for measurements on the right and left breasts.

The changes described in this chapter are around 20% of the minimum breast volume, the mean total change in normal cycles being 100 ml. The mean change in Pill cycles was 66 ml. There have been no previous studies on breast volume changes in response to oral contraceptives.

The results described here indicate that the change in breast volume is a response to progesterone rather than oestrogen. Volumes in the normal cycles are lowest around the 9th day of the cycle, when oestrogen concentrations are highest. Fig 3:1 and Fig 3:3 show that in contraceptive-controlled cycles the increase in volume begins earlier than in the

normal cycle - in a contraceptive-controlled cycle a progestogen is taken from day 7, whereas during a normal 28-day cycle progesterone appears in the circulation only after day 15.

The mechanism of the change in breast volume is not clear. The histological study described in Chapter 4 indicates that marked histological changes do not occur during the normal menstrual cycle. The most likely explanation of the volume changes is that of fluid retention, allied to vascular changes. During the normal menstrual cycle there are overall changes in the amount of fluid retained in the body tissues, but the total change in body weight during the cycle is no more than 1 kg in most women (Parboosingh et al 1973; Thorn et al 1938). The 20% change in breast volume is therefore not merely a reflection of generalised fluid retention.

Vascular changes in the breast have been studied by various methods. Thermography is employed clinically to study the vascular pattern of the breast, and Isard and Shilo (1968) performed weekly thermograms on ten healthy volunteers, aged 20-43. The parity of the subjects is not stated. Correlation of vascular changes with the cycle was poor in this study, but in five of the women there was increased vascularity during the week before menstruation. Parry et al (1972) studied nine healthy women by thermography: again the parity of the women is not recorded, but from the fact that they were student radiographers it seems likely that most of them were nulliparous. No cyclical changes were found in the thermograms. Parry et al attribute this negative finding to the fact that they compensated for the change in basal body temperature that occurs in the second half of the cycle: previous investigators had not compensated for this rise, and Parry et al suggest

that this explains the apparent changes found.

Zeppa (1969) studied the vascular response of the rabbit mammary gland to hormones, using a technique of vascular labelling of whole mounts of breast tissue. If the breast were pre-treated for two days or more with oestrogen, a submammary injection of oestrogen caused increased vascular permeability. This response was abolished by antihistamines. The response to other hormones such as progesterone was not studied.

Masters and Johnson (1966) reported a 20-25% increase in breast volume during intense sexual excitement, and attributed this change to vasocongestion. The method of measuring this change is not given, but if it is genuine then it demonstrates that vascular changes alone could explain this amount of volume change. The volume changes measured by Hytten (1954) during pregnancy are likely to have a different basis, being associated with glandular development during the mid-trimester of pregnancy (Chapter 2) and being correlated with the amount of milk yield during later lactation.

It is unfortunate that parous women were not also studied. Parous women report similar breast symptoms to those experienced by nulliparae (Chapter 4), but as yet there is no objective proof that their breast stroma responds to the cycle in the same way as the nulliparae studied in this chapter.

Summary

Four nulliparous women measured the volume of their right and left breasts daily during the normal menstrual cycle and during contraceptive-

controlled cycles. In both normal and contraceptive-controlled cycles the breast volume increased in the second half of the cycle. The increase was around 20% of the minimum volume, the mean change being 100 ml in normal cycles and 66 ml in contraceptive-controlled cycles.

CHAPTER FOUR

Chapter 4

HISTOLOGICAL STUDIES

I INTRODUCTION

The main purpose of this project was to study normal breast histology, in order to find out whether differences exist between nulliparous and parous women as far as the response of their breast tissue to the menstrual cycle is concerned. Certain difficulties are immediately obvious. It is not possible to take tissue from the same woman before and after pregnancy. Nor is it possible to obtain serial specimens of tissue from a woman during a single cycle (though this has been carried out in Rhesus monkeys (Speert 1948). Needle biopsy, used in the diagnosis of malignant disease, does not provide enough tissue for histological analysis.

Therefore, as discussed in Chapter 2, most investigators have found it necessary to obtain tissue either at biopsy of a breast lump or at post-mortem. Because there is much variation between individuals, large numbers of specimens are required, and these can only be accumulated by using biopsy material. The advantages of using such material are that a history can be obtained from the patient, blood can be taken for hormone estimation, and fresh tissue is obtained suitable for organ culture. The disadvantages are that only a single small specimen can usually be obtained, and that because a breast lump is present the breast cannot be regarded as truly normal. These points are discussed in more detail in Section IV of this chapter, which also contains an account of my attempt at using post-mortem material.

Despite the imperfections, therefore, there was no alternative to the use of tissue obtained at breast biopsy. The help of several surgeons (who are acknowledged elsewhere in the thesis) was therefore sought and was most willingly given.

II PATIENTS AND METHODS

Specimens of tissue were obtained from 174 patients. Of these, 156 were undergoing surgery for biopsy of a breast lump: these operations were performed in the Royal Infirmary (Wards 7 & 8), Chalmers Hospital, Longmore Hospital and the Deaconess Hospital, Edinburgh. The remaining 18 were undergoing reduction mammoplasty at Bangour Hospital, Broxburn, West Lothian. All the patients were interviewed by me before operation, except for five patients who were interviewed by the medical or nursing staff in my absence. The purpose of the study was explained to them, and they all agreed to allow normal tissue to be taken.

A History

The data form used for recording the patient's history is given in Appendix 1. The patient's age, age at menarche, parity and dates of pregnancies were recorded. The date of the last menstrual period was noted, along with the regularity of the menstrual cycle. A note was taken of the times that the patient had taken oral contraceptives in the past. In case anxiety over her condition might have upset the regularity of the menstrual cycle, a note was made of the date when she first saw a doctor about the breast lump. From 118 patients details were taken about premenstrual breast tenderness or other premenstrual symptoms. To check that the next period came at the expected date, 112 patients were asked to fill in a note giving the date of their next period after the operation, and post it to me in a prepaid envelope. The rate of return of these envelopes was high (103 out of 112: 92%). Patients taking oral contraceptives, and those who turned out to have cancer, were not asked to do this.

Tables 4:1 to 4:8 give a summary of the histories obtained.

TABLE 4:1

Age and menstrual age of patients studied

Patients studied	Total 174			
Age range ...	14 - 52			
Mean age ...		33.9	SD: 9.6	SE: 0.7
Menstrual age range ...	1 - 38			
Mean menstrual age ...		20.7	SD: 9.7	SE: 0.7
Parous patients	Total 115			
Age range ...	18 - 52			
Mean age ...		36.3	SD: 7.6	SE: 0.7
Menstrual age range ...	4 - 38			
Mean menstrual age ...		23.1	SD: 7.9	SE: 0.7
Nulliparae	Total 59			
Age range ...	14 - 52			
Mean age ...		29.2	SD: 11.3	SE: 1.5
Menstrual age range ...	1 - 37			
Mean menstrual age ...		16.3	SD: 11.3	SE: 1.5

"Menstrual age" means the age of the patient minus her age at menarche

SD means Standard Deviation

SE means Standard Error of Mean

In all subsequent Tables in this chapter, Standard Errors are given after the "±" sign, not Standard Deviations.

TABLE 4:2

Parity of patients:

		Numbers
Nulliparous	...	59
0 + 1	...	3
1 + 0	...	14
1 + 1	...	4
1 + 2	...	1
1 + 4	...	1
2 + 0	...	41
2 + 1	...	13
2 + 2	...	1
2 + 3	...	1
3 + 0	...	13
3 + 1	...	8
3 + 2	...	3
3 + 3	...	1
3 + 5	...	1
4 + 0	...	4
4 + 1	...	2
4 + 2	...	1
5 + 0	...	2
6 + 0	...	1
	TOTAL	174

The first figure refers to the number of pregnancies of more than 28 weeks' gestation. The second figure refers to the number of miscarriages (ie. less than 28 weeks' gestation).

TABLE 4:3a

Menstrual cycles of nulliparous patients studied (Total: 49)

Length of menses		Lengths of cycle		Irregular cycles	
2 days ..	1	23 days ..	1	21-28 days ..	1
3 days ..	2	24 days ..	0	14-45 days ..	1
4 days ..	11	25 days ..	2	28-56 days ..	1
5 days ..	25	26 days ..	1	5-7 weeks ..	1
6 days ..	4	27 days ..	0	21 days - 6 months ..	1
7 days ..	5	28 days ..	36		
10 days ..	1	29 days ..	0		
		30 days ..	2		
		33 days ..	1		
		35 days ..	1		

Nulliparae on oral contraceptives (Total 10)

Length of menses			
3 days ..	3		
4 days ..	5	28 day cycle ..	10
5 days ..	1		
7 days ..	1		

In Table 4:3a and Table 4:3b the length of cycle and length of menses are those given in the patient's history.

Table 4:4 shows the accuracy of the history as indicated by the letters patients sent notifying me of the date of their next period.

TABLE 4:3b

Menstrual cycles of parous patients studied (Total: 95)

Length of menses		Length of cycle		Irregular cycles	
2 days	2	21 days	8	14-21 days	2
3 days	9	22 days	0	14-42 days	1
4 days	10	23 days	1	14-49 days	1
5 days	34	24 days	1	14 days - 3 months	1
6 days	14	25 days	3	21-35 days	1
7 days	16	26 days	5	28-35 days	1
8 days	4	27 days	3	28-42 days	1
9 days	1	28 days	52	6-8 weeks	1
10-15 days	1	29 days	2	35-42 days	1
Not known	1	30 days	5	Post-menopause	2
3-7 days	1	35 days	2		
Post-menopause	2	3 months	1		

Parous patients on oral contraceptives (Total 20)

Lengths of menses			
1 day	1		
2 days	1		
3 days	3	26 day cycle	1
4 days	11	28 day cycle	18
5 days	1	On "tricycle pill"	1
6 days	2		

TABLE 4:4

Confirmation of stage of cycle

Days early compared with prediction	Nulliparae (Total: 36)	Parous patients (Total: 60)
20	1	
18		1
10	2	1
8	1	
7		2
6	1	
5		
4		2
3		5
2	2	11
1	4	7
On time	8	11
Days late compared with prediction		
1	2	5
2	3	1
3	3	2
4	4	1
5		4
6		3
8	1	1
9	1	
10	1	
15		1
16	1	
20	1	
25		1
5 months		1

TABLE 4:4 (continued)

3 Nulliparae and 2 parous patients on oral contraceptives confirmed that their periods arrived at the right time. One parous patient on oral contraceptives had a period one day late. One nullipara confirmed that she was post-menopausal.

TABLE 4:5

History of oral contraceptive ("Pill") use

"Pill"refers to combined oestrogen-progestogen oral contraceptives, of various types.

Never on Pill	.....	83
On Pill in past	.....	63
Less than 1 year	.. 28	
1-2 years	.. 15	
2-4 years	.. 12	
5 years	.. 3	
6-8 years	.. 3	
8-10 years	.. 1	
Unknown	.. 1	
	63	
Currently on Pill	.....	28
Less than 1 year	.. 6	
1-2 years	.. 6	
2-4 years	.. 4	
5 years	.. 4	
6-8 years	.. 4	
8-10 years	.. 0	
More than 10 years	.. 2	
Unknown	.. 2	
	28	
Total	..	174

TABLE 4:6 Breast Feeding History in 112 Parous Patients

Not known	10
No breast feeding	42
Breast feeding	60
Fed first baby but not second	13

Fed one baby only	
2 days	1
3 days	1
1 week	1
10 days	1
2 weeks	4
3 weeks	2
4 weeks	2
"a few weeks"	4
6 weeks	3
2 months	1
3 months	2
3½ months	1
4 months	1
5 months	1
7 months	1
8 months	1
9 months	1
TOTAL	28

Fed more than one baby	
1 week & 2 weeks	1
2 weeks & 1 week	1
1 month x 4	1
6 weeks & 2 weeks	1
6 weeks/0/6 weeks/0	1
6 weeks & 2 weeks	1
6 weeks x 3	1
3 months & 6 weeks	1
3 months & 2 months	1
3 months x 2	3
0/3 months x 2	1
3 months & 4½ months	1
4 months/2 months x 2	1
5 months x 2/2 months	1
5 months & 6 months	1
6 months & 2 months	1
6 months & 3 months	1
6 months & 3 months x 2	1
6 months & 6 months	1
6 months/4½ months/1 month	1
7 months & 2 months	2
7 months & 4 months	1
7 months x 2	1
7 months x 3	1
8 months & 6 months	1
8 months x 2	1
0 & 9 months	1
9 months & 6 weeks	1
9 months & 8 months	1
TOTAL	32

TABLE 4:7 Menstrual symptoms

a) Breast symptoms during the cycle among 115 patients asked

Tenderness (91 patients were specifically asked about this)		
None	42	
1-2 days before menses	14)	
3 days - 1 week before menses	30)	49
1-2 weeks before menses	5)	
Swelling		32
Swelling only on Pill	1	
Lumpiness		1
Mastitis		1

b) OTHER Menstrual Symptoms

Pre-menstrual tension (96 patients specifically asked)		
None	43	
1-2 days before menses	27)	53
4 days - 1 week before menses	26)	
Abdominal pain		5
Depression		4
Headache		3
Irritability		2
Tiredness		1
Various symptoms		1
"Feels great" (2 days before menses)		1

"Swelling" includes: bloatedness, heaviness, fullness, hard, and tight

(Totals are not given because some patients had more than one symptom. For totals see Table 4:8)

TABLE 4:8

Correlation between breast symptoms and pre-menstrual tension (PMT)

Neither	24
Breast symptoms but no PMT	31
PMT but no breast symptoms	18
Both	42
TOTAL	115

	No PMT	PMT	
No breast symptoms	24	18	42
Breast symptoms	31	42	73
	55	60	115

Correlation: X^2 : 1.75 Not significant

B Hormone estimation

A sample of 10 ml of venous blood was taken either at the time that the patient was interviewed (which was within 24 hours of operation) or by the anaesthetist during the operation. Blood was centrifuged and the plasma was stored in the deep freeze ($-20^{o}C$) within a few hours of being obtained.

Plasma progesterone was estimated by radioimmunoassay using a modification of the method of Neal et al (1975).

Plasma oestradiol was estimated by radioimmunoassay using a modification of the method of Cameron et al (1972).

C Biopsy technique

All biopsies were taken under general anaesthesia. Since many different anaesthetists were involved over the series of 174 patients, the techniques of anaesthesia varied.

Through a single incision, first the clinically abnormal lump was removed and sent for examination by the pathologist. Then a piece of macroscopically normal tissue was removed by the surgeon from a site as far away as possible from the clinically abnormal lump.

With reduction mammoplasty specimens, glandular tissue was selected from the excised tissue soon after its removal. The selection was usually random, though in some cases it was possible to record the quadrant from which the tissue was taken.

With biopsy specimens, the site of the biopsy was checked by noting the side, quadrant, and distance from the nipple, of the scar after operation.

The dates and sites of the biopsies are given in Tables 4:9 and 4:10. From some patients multiple specimens were taken, and details of these are given in Table 4:11.

TABLE 4:9 Day of cycle on which biopsy was taken (according to menstrual history)

Day	Nulliparae	Parous (including 0+1)
0	1	4
1	1	1
2	0	5
3	1	2
4	2	6
5	1	2
6	2	3
7	3	4
8	2	4
9	1	7
10	3	7
11	3	0
12	2	4
13	5	7
14	1	5
15	1	5
16	2	6
17	1	3
18	0	3
19	3	4
20	1	3
21	1	3
22	2	2
23	3	2
24	4	3
25	0	3
26	1	2
27	1	3
28	1	3
29	2	0
30	1	0
31	0	1
32	0	2
33	1	0
37	1	
39	1	
Not known	1	3
Post-menopausal	2	2
Pregnant	1 Tricycle pill	1
	59	115

TABLE 4:10

Plasma progesterone concentrations during the cycle in parous and nulliparous women (ng/ml)

Day of cycle	Nulliparae	Parous women
1 - 7	0.460 ± 0.199 (n: 7)	0.968 ± 0.298 (n: 23)
8 - 14	1.460 ± 0.589 (n: 14)	1.688 ± 0.517 (n: 28)
15 - 21	6.165 ± 1.724 (n: 6)	6.452 ± 1.011 (n: 21)
22 - 28	8.921 ± 1.734 (n: 7)	7.010 ± 1.261 (n: 16)
29+	6.993 ± 2.976 (n: 4)	7.933 ± 5.118 (n: 3)

During the follicular phase of the cycle, 7 of the 21 nulliparae and 15 of the 51 parous women had plasma progesterone concentrations greater than 1 ng/ml (3.2 n mol/1).

During the luteal phase of the cycle, 1 of the 13 nulliparae and 3 of the 37 parous women had plasma progesterone concentrations less than 1 ng/ml (3.2 n mol/1)

TABLE 4:11

Plasma oestradiol-17β concentrations during the cycle in parous and nulliparous women (pmol/1)

Day of cycle	Nulliparae	Parous women
1 - 7	530 ± 141 (n: 8)	363 ± 53 (n: 21)
8 - 14	572 ± 99 (n: 14)	737 ± 104 (n: 24)
15 - 21	413 ± 94 (n: 4)	593 ± 63 (n: 17)
22 - 28	597 ± 169 (n: 6)	418 ± 78 (n: 11)
29+	423 ± 153 (n: 4)	346 ± 227 (n: 2)

In this and the previous table, the "day of cycle" is that given by the menstrual history.

TABLE 4:12 Sites of Biopsies

a) Quadrant	Nulliparae	Parous patients
R upper outer	12	22
R upper inner	3	12
R lower outer	5	3
R lower inner	0	3
L upper outer	12	15
L upper inner	3	11
L lower outer	2	3
L lower inner	2	8
Not known	12	33
Multiple specimens	8	5
TOTAL	59	115

b) Distance from nipple		
1 cm	2	8
2 cm	8	14
3 cm	2	15
4 cm	4	10
5 cm	5	10
6 cm	15	13
7 cm	1	2
8 cm	0	1
Not known	14	37
Multiple specimens	8	5
TOTAL	59	115

TABLE 4:13

Relationship between site of biopsy and parity

Distance from nipple (cm)	Nulliparae	Parous women
1	2	8
2	6	15
3	2	16
4	6	10
5	6	12
6 or more	13	16
Total 114	37	77
Quadrant of breast		
upper/outer	23	35
upper/inner	8	24
lower/outer	4	8
lower/inner	1	15
Total 118	36	82

The distribution is not significantly different among parous women and nulliparae.

TABLE 4:14 Patients from which multiple specimens were obtained

Parity	Diagnosis	Area biopsied	Number of specimens
Parous	Fibroadenoma	R & L breasts	2
Parous	Fibrocystic disease	R upper inner quadrant	2
Nullip	Fibroadenomata	R & L breasts	2
Nullip	Mammoplasty	R med & lat sides	2
Nullip	Mammoplasty	R med & lat sides	2
Nullip	Mammoplasty	R med & lat sides	2
Nullip	Mannoplasty	R & L breasts	2
Parous	Fibroadenomata	R & L breasts	2
0+1	Mammoplasty	4 from each breast	8
Parous	Mammary dysplasia	R & L upper outer quadrants	2
Nullip	Mammoplasty	3 from each breast	6
Nullip	Fibroadenomata	R & L breasts	2
Nullip	Mammoplasty	2 from each breast	4
Parous	Mammoplasty	5 from one breast	5
Nullip	Mammoplasty	10 from one breast: 2 central and 2 from each of four quadrants (one from central and one from peripheral part of each quadrant)	10

(Normally with mammoplasty specimens it was impossible to identify the quadrant or distance from the nipple from which the tissue was obtained, as tissue was taken randomly from the specimens removed by the surgeon).

The size of the biopsy of normal tissue varied but it was usually a cube of around 0.5 to 1.0 cm side. It was immediately placed in 4% neutral buffered formaldehyde.

At the same time smaller specimens were obtained from most of the patients for fixation in glutaraldehyde, and samples of tissue were frozen on solid CO_2.

Some of the patients also provided specimens for the study of DNA synthesis and immunoglobulin synthesis. This tissue was obtained at the same time and was transported separately in different media, as described in Chapter 5 and Chapter 6.

D Diagnosis of the breast lump

The histopathology of the primary condition for which the biopsy was undertaken is noted in Table 4:15. The diagnoses in this table are those made by the routine pathology services to the various hospitals. The diagnostic labels attached to the benign conditions vary considerably, and in Table 4:15 they have been grouped into three groups. Group one consists of conditions involving a discrete lump such as a fibroadenoma or a lipoma - which may be expected to leave the rest of the breast entirely normal. This group also includes the eighteen patients who had normal tissue removed at mammoplasty - the pathologist checked this tissue in each case and reported it as normal. Group one also includes four cases in which the apparent lump removed consisted of normal tissue.

Group 2 consists of benign conditions which may involve more than one area of the breast (for example, fibrocystic disease).

Group 3 consists of eleven patients who proved to have carcinoma. These were excluded from further analysis.

TABLE 4:15 Histopathology of primary condition

Group 1: Localised conditions

Fibroadenoma	36
Cyst and papilloma	1
Lipoma	3
Simple cyst	1
Normal tissue	4
Mammoplasty	18
Dilated duct	1
Chronic abscess	1
Normal lymph node	1
TOTAL	66

Group 2: Fibrosing adenosis with or without cyst formation

Fibrocystic disease	17
Cystic disease and fibroadenosis	1
Fibroadenosis and fibrocystic disease	1
Fibroadenosis	43
Fibroadenosis and cysts with epitheliosis	1
Fibroadenoma and fibroadenosis	4
Fibroadenosis with cysts	3
Mammary dysplasia	4
Fibrosing adenosis	2
Fibroadenosis with focal epitheliosis	1
Sclerosing adenosis	1
Fibrosis	1
Cystic disease	1
Sclerosing fibroadenosis	1
Chronic inflammation	1
Cystic epithelial hyperplasia	1
Lobular adenosis	1
Fibrosis and cyst formation	1
Adenosis	1
Fibroadenomata with epithelial hyperplasia	1
Sclerosing adenosis with microcysts	1
Epitheliosis	1
Epitheliosis and adenosis	1
Mammary dysplasia (multifocal duct papilloma, fibroadenosis, fibrosing adenosis, focal epitheliosis)	1

TABLE 4:15 (continued)

Adenosis, fibrosis, epitheliosis, sclerosing adenosis	1
Interlobular fibrosis, fibroadenosis, involution	1
Fibrosclerosis and atypical lobular hyperplasia	1
Fibroadenoma and cystic epithelial hyperplasia	1
Cystic epithelial hyperplasia and duct ectasia	1
Epitheliosis and sclerosis with adenosis	1
TOTAL	97

Group 3: Malignant disease

Carcinoma	11
TOTAL	174

TABLE 4:16

Relationship between parity and diagnosis of the primary benign condition

	Group 1 (localised disease)		Group 2 (possibly generalised disease)	
Nulliparae (n: 56)				
Proliferative phase	16)		12)	
Luteal phase	10)	29	8)	27
On Pill	3)		7)	
Parous women (n: 107)				
Proliferative phase	16)		39)	
Luteal phase	11)	37	24)	70
On Pill	10)		7)	
TOTAL	66		97	

See text for explanation of "Group 1" and "Group 2".

There is no significant difference between parous and nulliparous women as regards the type of breast disease. There is no difference between the phases of the cycle as regards the type of disease.

III HISTOLOGY

A Preparation of the sections

The tissue blocks were stored in 4% neutral buffered formaldehyde for several days until a group of about ten specimens was collected. Sections 4μ thick were then cut on a standard rotary microtome. Multiple sections were taken from some blocks (for comparison with one another) but normally only one series of sections, each stained differently, was kept from each specimen.

Cutting and staining of the sections were carried out under the supervision of Mr R R Hogg of the Research Laboratory of the Department of Pathology.

Most of the histological analysis (except where stated) was carried out on sections stained with haematoxylin and eosin. This staining was performed by the standard method (Drury and Wallington 1967, p 129) in which nuclei appear blue to blue-black, cytoplasm appears as shades of pink, and fibrous and elastic tissue also appears pink.

Other stains used were as follows:

Methyl Green-Pyronin: (Drury and Wallington 1967, p 160)
This method uses a mixture of two basic dyes to stain chromatin and basophilic inclusion bodies (usually RNA). DNA is stained green or blue-green, and RNA granules stain dark rose red. The cytoplasm of plasma cells stains purple.

Gomori's Aldehyde Fuchsin Stain (Drury and Wallington 1967, p 179). This method shows elastic fibres (and also some mucopolysaccharides and mast cell granules) as a deep purple-violet.

Alcian Blue Stain for acid mucopolysaccharides (Drury and Wallington 1967, p 212). This method, in which acid mucopolysaccharides appear

blue, was combined with the Periodic Acid-Schniff method (Drury and Wallington 1967, p 204) in which neutral polysaccharides appear red or magenta.

Best's Carmine Method for Glycogen (Drury and Wallington 1967, p 208). This stain shows glycogen as bright red granules, while nuclei stain blue.

Masson's Trichrome Stain (Drury and Wallington 1967, p 167). This stain for collagen shows nuclei as black, fibrin as red, and collagen (using the light green counterstain) as green. Reticulin and mucin also stain green.

Enzyme methods were also used on tissue which had been frozen on solid CO_2 after removal, but satisfactory staining was not obtained and so no analysis was carried out of this material.

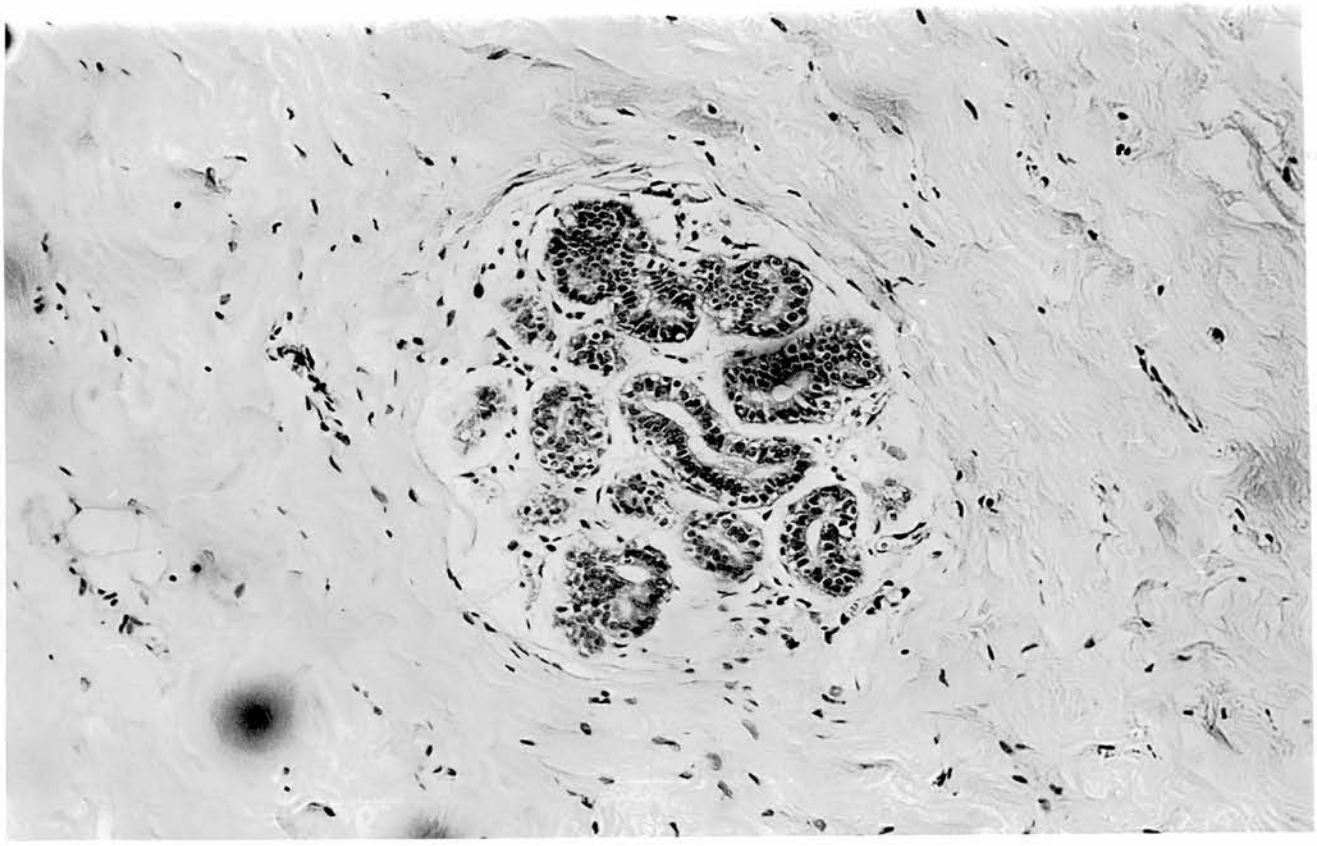
Histological Analysis

All the histological analysis was carried out by myself. Dr I I Smith and Dr T J Anderson of the Department of Pathology gave helpful advice.

x 80

Fig 4:1(a) To illustrate a normal lobule

Mammoplasty specimen obtained from a 21 year-old nulliparous woman on the tenth day of a 26-day cycle. The plasma progesterone concentration was "undetectable". Her menarche had been at the age of 11. Objectively the breasts did not appear grossly over-developed, and she had no breast symptoms, but she said that breast development had begun at the age of 7, two years after radiotherapy to a tumour of the orbit.



x 200

Fig 4:1(b) To illustrate a normal lobule.
This is a second normal lobule from the same section as that illustrated in Fig 4:1(a). Intralobular stromal oedema is clearly seen.

The histological analysis is divided into three broad areas: the breast lobules, the ductules, and the stroma (both intralobular and extralobular). There is some overlap between these three headings, but it convenient to describe them separately.

B Examination of the lobules

1 Methods

The structure of a typical breast lobule is shown in Fig 4:1(a & b). The lobule consists of a number of ductules (also called acini) embedded in a stroma of loose connective tissue. The ductules arise from a terminal ductule which can rarely be identified in a section. The loose connective tissue (the intralobular stroma) is usually clearly distinguishable from the remainder of the breast stroma (the extralobular stroma - also called the interlobular stroma). The variables examined were as follows.

a) Lobules per unit area The number of lobules in each section was counted. A lobule was defined as three or more ductules in close apposition. The area of the section was measured using a measuring eyepiece with a scale. (The units in this scale measured 3.0 mm in length when used with the lowest-power objective lens (4/0 . 12). The length and breadth of the section were each measured, and multiplied together to obtain the area. Although sections were not exactly rectangular, making this method of measurement slightly inaccurate, it is felt to be sufficiently accurate for its purpose, which is comparison of sections with one another. The areas were measured in square millimetres.

b) Ductules per unit area The number of ductules in each lobule was counted. The total number of ductules in the section was obtained by addition, and was divided by the area of the section. When the

number of lobules in the section was much greater than twenty, the number of ductules in the first twenty lobules was counted, and the total number of ductules was obtained by multiplying the total number of lobules by the average number of ductules in the first twenty lobules.

c) Percentage area of lobules This figure represents the proportion of the total section area occupied by lobules. Two methods were used to obtain this value: direct measurement and automated analysis.

(i) Direct measurement - The dimensions of each lobule in a section were obtained using the measuring eyepiece. The area of each lobule was obtained by multiplying its length by its breadth - involving an approximation since the lobules are not rectangular. The areas of all the lobules were added up to give the total area occupied by the lobules, which was then divided by the total area of the section (found by direct measurement as described above) to give a percentage. This was a time-consuming method, and was used in 21 sections altogether.

(ii) Automated analysis - Because measurement of each lobule was laborious, an alternative method was sought. Automated image analysis was available from a commercial research organisation, Inveresk Research International, using a "Quantimet" image analyser. The"Quantimet" (Imanco, Cambridge Instruments Ltd, Melbourn, Royston, Herts) is a machine which consists of a video screen connected to a microscope. Connected to the video screen is a computer which can give a point count, using a grid on the screen of 600 x 450 - ie. 270,000 - points. Detection levels can be set, and in this case two levels were used: dark areas

indicated glandular material in the section of breast tissue being scanned, and the lighter areas were stroma. The method depended on the fact that the glandular tissue is much more cellular than the stroma, and the concentrations of darkly-staining nuclei made the glandular tissue appear dark on the black-and-white video screen. At the magnification and resolution used, the point count was a count of whole cells rather than nuclei only. Only low-power scanning was carried out, and a green filter was used to clarify the black-and-white image. Approximately 10 to 20 fields were scanned in each specimen, and the whole specimen was included. The smallest section required three fields, and the largest 40, with a mean of 17. Only haematoxylin and eosin sections were used, and the analysis was done by myself after suitable training in the use of the machine.

A comparison between the results of Quantimet analysis and direct measurement of the lobules is given in the Results section.

d) Average number of ductules per lobule This was found by counting the ductules in each lobule. When a section contained a very large number of lobules (more than 25), only the first 20 lobules were examined. A lobule was defined as being separate from an adjacent lobule if a distinct line of extralobular stroma could be seen between them.

e) Area of average lobule This was obtained by measurement (as described in (c) above) of the total area of the lobules: this figure was divided by the number of lobules, obtained by direct counting. The result was expressed in square millimetres.

f) Ductule density in lobules This figure represents the number of ductules per unit area of the lobule. It is expressed in ductules per square millimetre. It was obtained by dividing the average number of ductules per lobule ("d", above) by the area of the average lobule ("e", above).

2 Results

a) Variation

(i) Quantimet analysis

(α) Reproducibility of Quantimet analysis

When the same section was examined by the "Quantimet" on different days, some variation in the readings was noted. This variation is shown below (Table 4:17).

TABLE 4:17 Variation in "Quantimet" readings of lobule area, between examinations on different days

Slide no.		Point counts: Points on Lobules	Points on Stroma	% area of lobules
21	Reading on 1st day	244,853	6,993,245	3.5
	Reading on 2nd day	346,284	5,319,378	6.5
50	Reading on 1st day	144,753	1,561,587	9.3
	Reading on 2nd day	143,770	1,143,894	12.6
80	Reading on 1st day	68,842	1,963,211	3.5
	Reading on 2nd day	42,681	1,501,240	2.8

(r for % area of lobules = 0.93)

The variation between the two readings on the same section on different days occurs because of difficulty in setting detection levels on the "Quantimet" in exactly the same way at each session. It was not possible to examine all the sections in the same session, but in an effort to minimise the variation the whole series of 100 sections was examined in three sessions.

(β) Variation between specimens from the same breast

Three pairs of specimens, each pair from a single breast, were examined using a "Quantimet", and the results are shown below (Table 4:18).

TABLE 4:18

"Quantimet" analysis - variation between specimens from a single breast

Section number	% area of lobules	
	First specimen	Second specimen
53	1.0	0.4
74	8.4	29.4
92	10.7	8.7

r= 0.54 (NS)

The variation in this series of three pairs is fairly marked. This is considered further later in this section.

(γ) Relation between lobule area measured by "Quantimet" and measured directly

In nine sections the lobule area was measured directly as described in the Methods section (part c(i), page 4-30), and also by the automated method. The correlation between these two methods is shown in Fig 4:2. The correlation coefficient, r, = 0.807. This correlation is unsatisfactory, indicating that one or both methods is subject to an appreciable degree of error. However, it indicates that the automated analysis gives a guide, if not an accurate measurement, of the proportion of glandular tissue present.

(ii) Variation in one block - Several sections from a single block were examined in three cases to assess the amount of variation over a small area of tissue. The blocks were chosen by a colleague (TJA) to represent different groups of patients,

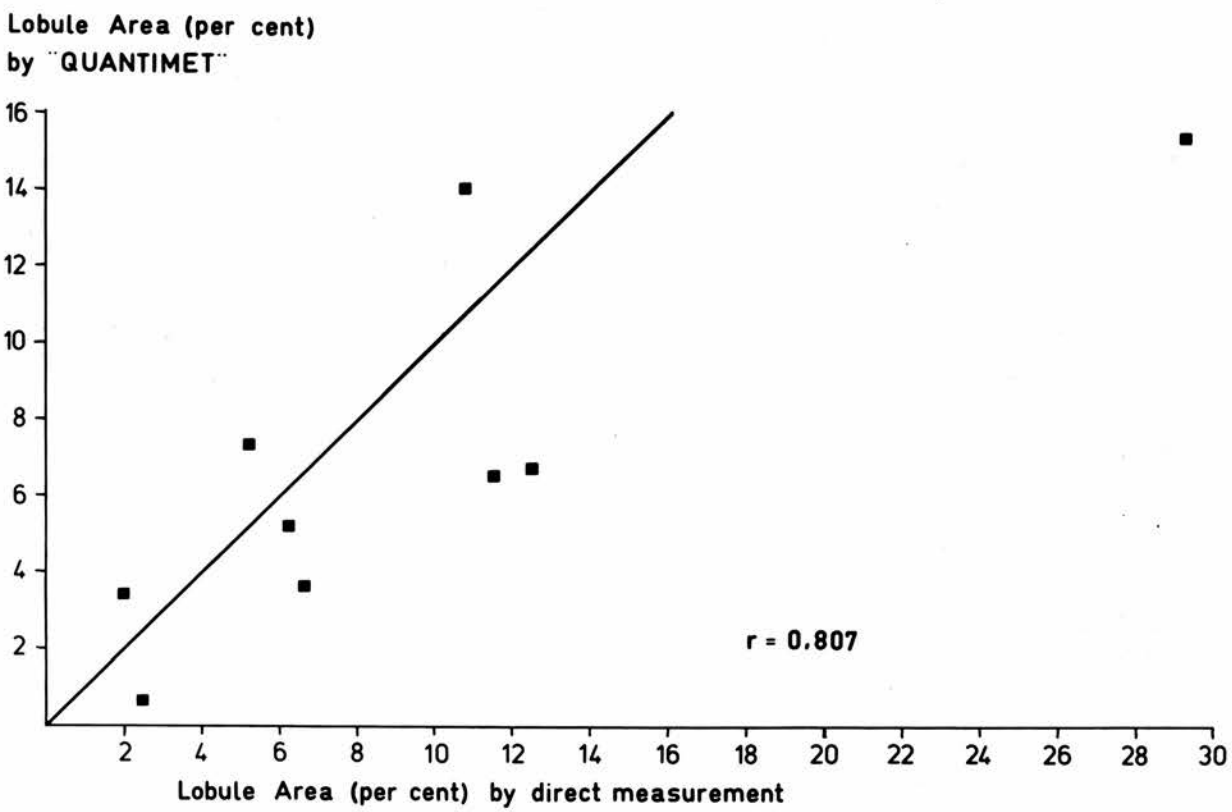


Fig 4:2: Lobule area (per cent). Relationship between direct measurement and measurement by "Quantimet" automated analysis.

but were examined "blind" after relabelling. Specimen 171 is from a nullipara, never on the Pill, at day 13 of her cycle. Specimen 101 is from a parous woman, on the Pill in the past but not at the time of biopsy, at day 28. The results are presented overleaf (Table 4:19).

Table 4:19 shows that there is substantial variation between sections cut from the same block (although these sections were not adjacent: every tenth section was examined). Despite this variation, the difference between patients was still greater than the difference between sections, and the asterisks in Table 4:19 denote significant differences between specimens 101 and 171 for the first four factors examined - ie. for all parameters except the area of the average lobule and the ductule density within the lobules, both of which seem constant among different patients.

(iii) Variation in one breast - To asses the amount of variation between different areas of a single breast, ten blocks were taken from one breast at mammoplasty. Two blocks were taken from close to the nipple, and two from each quadrant - one from the periphery and one more central in each case. The results are presented in Table 4:20

TABLE 4:19 Variation between sections from a single block : Assessment of lobules

	Specimen Number	101			171		184	
		Minimum	Maximum		Minimum	Maximum	Minimum	Maximum
1	Lobules/sq mm	0.64	1.02	*	0.11	0.32	0.06	0.19
2	Ductules/sq mm	7.9	17.3	*	0.8	2.5	0.4	2.2
3	% area of lobules	7.0	19.7	*	0.7	2.7	0.7	1.0
4	Average number of ductules/lobule	10.0	19.4	*	5.7	10.4	6.3	11.4
5	Area of average lobule (sq mm)	0.08	0.23		0.05	0.12	0.05	0.11
6	Ductule density in lobules (/sq mm)	86	141		74	124	56	215
	Number of sections examined	10			8		2	

TABLE 4:20 Variation between different blocks from a single breast: Assessment of lobules

AREA OF BREAST	CENTRAL		INNER				OUTER				MEAN ± SE
	1	2	Upper Medial	Upper Lat	Lower Med	Lower Lat	Upper Med	Upper Lat	Lower Med	Lower Lat	
1 Lobules/sq mm	0.11	0.17	0.11	0.32	0.19	0.17	0.19	0.67	0.32	0.34	0.26 ± 0.05
2 Ductules/sq mm	1.5	1.9	0.9	4.8	2.8	2.8	2.7	12.9	4.6	4.0	3.9 ± 1.1
3 % Area of lobules	1.7	0.7	0.4	2.8	1.3	1.1	1.0	5.4	1.9	1.4	1.8 ± 0.5
4 Average number of ductules/lobule	15.0	12.2	10.3	14.6	15.6	16.2	16.9	19.4	14.0	11.8	14.6 ± 0.8
5 Area of average lobule (sq mm)	0.17	0.05	0.05	0.09	0.07	0.06	0.06	0.08	0.06	0.04	0.07 ± 0.01
6 Ductule density in lobules (/sq mm)	87	212	219	168	222	261	281	236	243	291	222 ± 19

TABLE 4:21 Assessment of lobules
Variation between right and left breasts

Patient number	8 (Parous, Follic phase)		51 (Nullip, on Pill)		94 (Nullip, Lut phase)	
	Right	Left	Right	Left	Right	Left
Lobules/sq mm	1.33	0.54	0.14	0.25	0.17	0.18
Ductules/sq mm	46.6	16.5	2.2	2.4	1.8	2.2
% Area of lobules	10.1	9.6	2.9	7.5	2.6	3.2
Average number of ductules/lobules	35	30.6	15.5	9.4	10.3	12.2
Area of average lobule (sq mm)	0.09	0.15	0.21	0.30	0.15	0.18
Ductule density in lobules (/sq mm)	398	201	75	31	69	68

Table 4:20 shows that the size of lobules and numbers of ductules per lobule are relatively constant, but that there is more variation in other parameters. In particular, there is a greater number of lobules and ductules per square millimetre in the upper outer quadrant of this breast. The question of the influence of the site of biopsy is discussed further in Section c(v).

(iv) Variation between right and left breasts - Bilateral biopsies were taken in three cases and the right and left breasts were compared. The results are presented in Table 4:21. From this table it appears that although there may be variation between the right and left breasts of one subject, this is less than the variation between different subjects.

Extreme examples of the variation between subjects are shown in Figs 4:3 and 4:4

b) Effects of parity and the menstrual cycle

Parity A comparison of the lobules between nulliparous and parous women is shown in Table 4:22. Most of the aspects studied show no significant difference between the two groups, although there is a trend towards higher numbers of ductules/sq mm among the parous women. However, significant differences were seen in two parameters: the number of lobules per unit area, and the ductule density in the lobules. In both these instances the significance of the difference was not high, P being just less than 0.05. Both, however, showed a similar trend with the higher values being found among the parous women (a similar trend to that found for the numbers of ductules/sq mm).

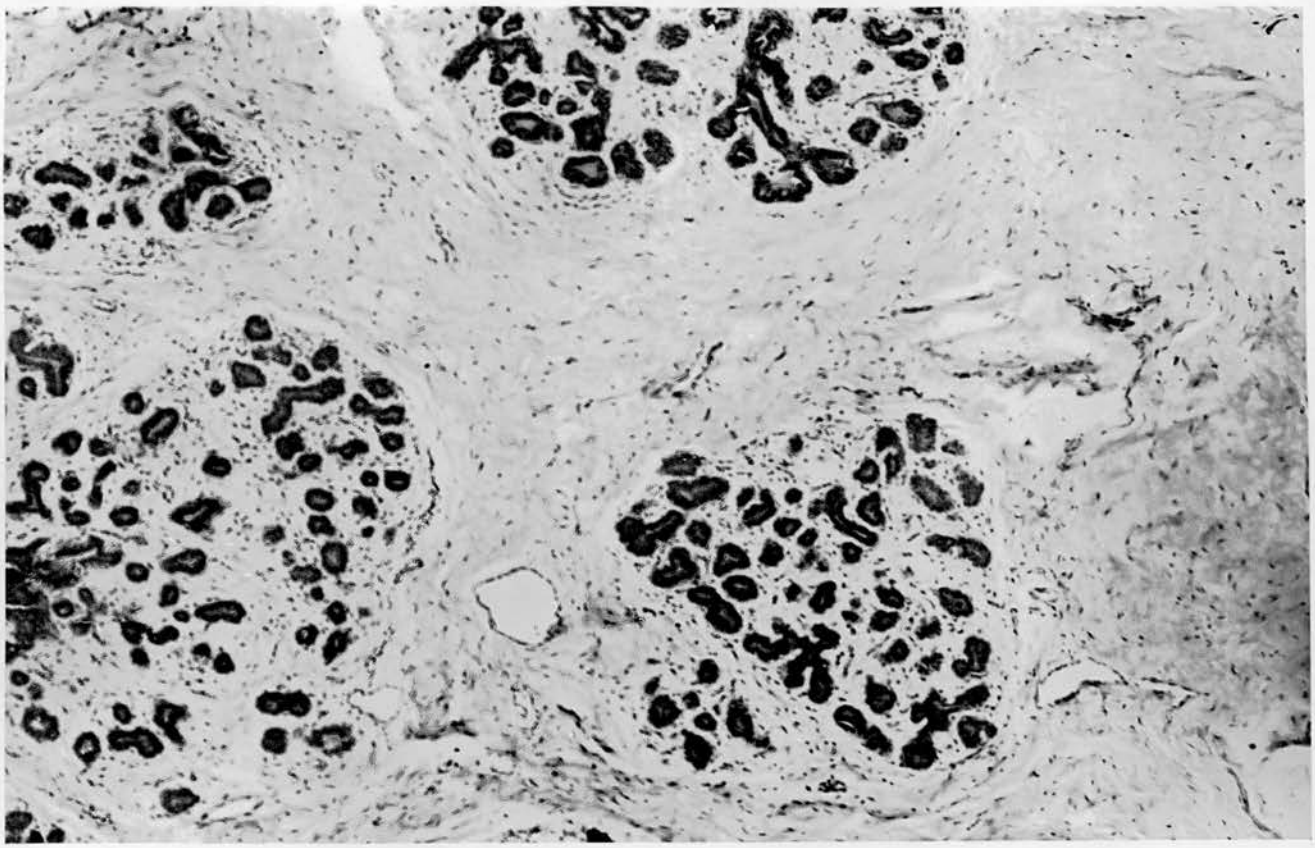


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Fig 4:3 To illustrate a specimen with large numbers of ductules/sq mm

The specimen was obtained at biopsy (diagnosis of lump: fibroadenosis) from a 45 year-old woman on the 12th day of a 21-day cycle. The plasma progesterone concentration was 7.4 ng/ml. Her menarche had been at the age of 14. She had had two full-term pregnancies, one when she was aged 27 and one at the age of 30. She had breast-fed for two weeks and one week respectively. She had always noticed premenstrual breast tenderness.

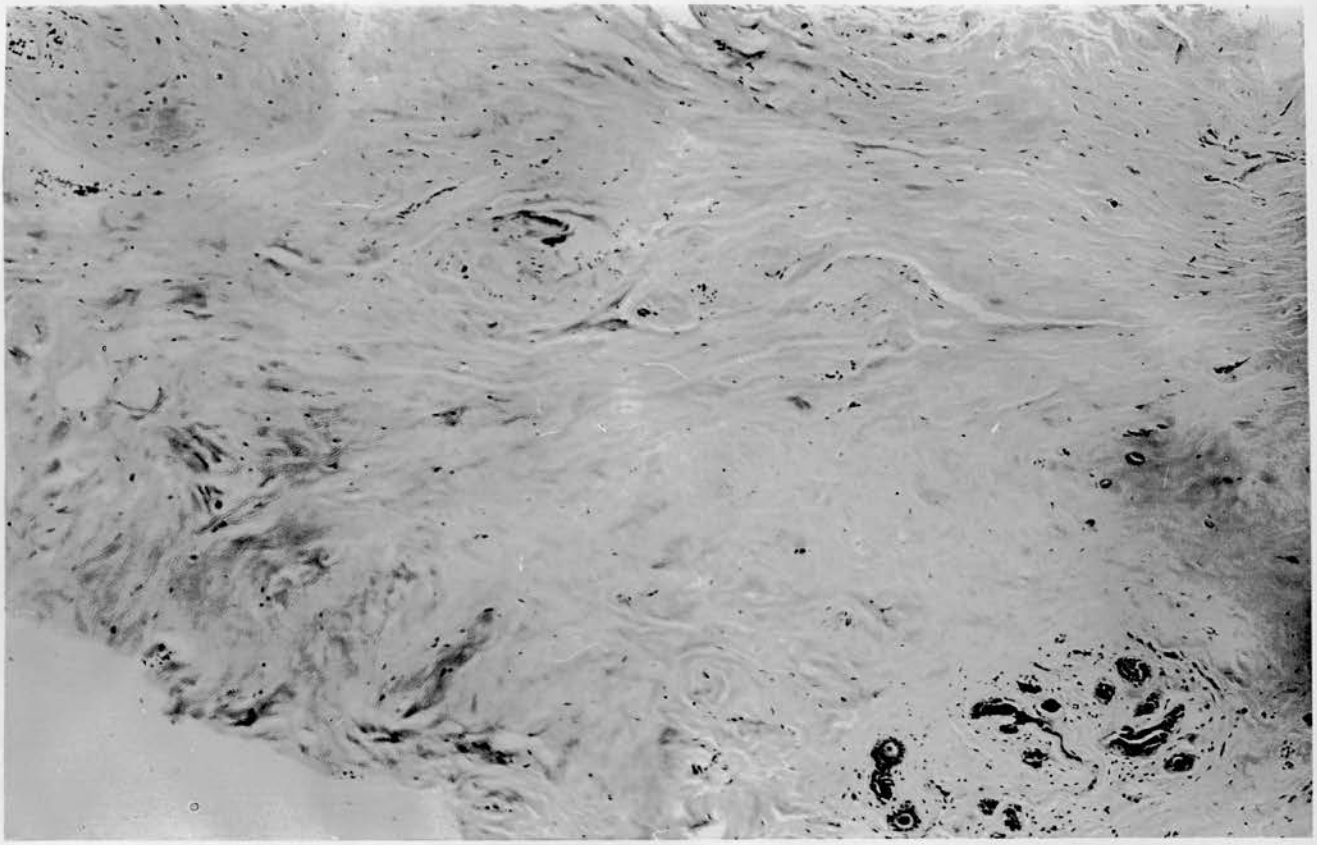

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Fig 4:4 To illustrate a specimen with small numbers of ductules/sq mm The specimen was obtained at biopsy (diagnosis of lump: fibroadenoma) from a 46 year-old woman on the eighth day of a 25-day cycle. The plasma progesterone concentration was "undetectable". Her menarche had been at the age of 11. She had had one full-term pregnancy at the age of 23, and one miscarriage. She had breast-fed for eight weeks.

TABLE 4:22 Assessment of lobules: effect of parity

	ALL NULLIPARAE		ALL PAROUS WOMEN
Lobules/sq mm	0.287 ± 0.049 (n: 40)	- P<0.05 -	0.428 ± 0.036 (n: 87)
Ductules/sq mm	6.05 ± 1.18 (n: 36)	- NS -	9.1 ± 1.06 (n: 67)
% area of lobules	8.23 ± 1.41 (n: 31)	- NS -	7.50 ± 0.66 (n: 62)
Average number of ductules/lobule	20.7 ± 2.2 (n: 34)	- NS -	22.0 ± 1.1 (n: 66)
Area of average lobule (sq mm)	0.227 ± 0.026 (n: 24)	- NS -	0.229 ± 0.025 (n: 62)
Ductule density in lobules (/sq mm)	112 ± 20 (n: 22)	- P<0.05 -	178 ± 19 (n: 45)

In this and subsequent Tables showing Mean ± Standard Error, statistical analysis was by Student's "t" test.

Menstrual cycle and oral contraception

Separate studies were made of women taking oral contraceptives, women in the follicular phase of the cycle, and women in the luteal phase of the cycle (as shown by a plasma progesterone concentration of over 1 ng/ml). Nulliparous and parous women were examined separately. The results are summarised in Table 4:23.

There is no significant difference between the phases of the cycle in either parous or nulliparous women, for any of the parameters studied. Nor do women taking oral contraceptives show any significant difference from either those in the follicular phase or those in the luteal phase.

Coded specimens

To eliminate possible bias in assessment of the specimens, 20 sections were selected by a colleague (TJA) and relabelled. The sections were selected to represent approximately equal numbers of parous and nulliparous women in each phase of the cycle. Assessment was then carried out by myself, "blind", and the code was not broken until the assessment was complete.

The results are set out in Tables 4:24 and 4:25.

TABLE 4:23 Assessment of lobules

Effects of parity, stage of cycle and oral contraception

	NULLIPARAE			PAROUS WOMEN		
	On Pill	Prolif phase	Luteal phase	On Pill	Prolif phase	Luteal phase
Lobules/sq mm	0.35 ±0.15 (n:10)	0.27 ±0.06 (n:16)	0.27 ±0.07 (n:14)	0.37 ±0.07 (n:12)	0.41 ±0.06 (n:43)	0.47 ±0.05 (n:32)
Ductules/sq mm	4.7 ±1.9 (n:8)	8.3 ±2.4 (n:14)	4.6 ±1.5 (n:14)	7.4 ±2.0 (n:10)	9.2 ±1.8 (n:35)	9.8 ±1.1 (n:22)
% area of lobules	8.29 ±3.63 (n:8)	8.77 ±2.15 (n:10)	7.77 ±2.11 (n:13)	7.23 ±2.46 (n:7)	7.55 ±0.99 (n:29)	7.84 ±0.97 (n:26)
Average number of ductules/lobule	20.27 ±4.81 (n:8)	26.54 ±4.45 (n:12)	15.9 ±4.44 (n:14)	21.93 ±2.87 (n:9)	22.44 ±1.81 (n:35)	21.28 ±1.51 (n:22)
Area of average lobule (sq mm)	0.221 ±0.051 (n:6)	0.238 ±0.049 (n:9)	0.220 ±0.039 (n:9)	0.358 ±0.101 (n:8)	0.213 ±0.041 (n:28)	0.195 ±0.025 (n:26)
Ductule density in lobules (sq mm)	121 ±43 (n:7)	113 ±35 (n:6)	103 ±31 (n:9)	135 ±61 (n:6)	178 ±24 (n:22)	189 ±35 (n:17)

There are no significant differences between the subgroups.

TABLE 4:24 Assessment of lobules

Coded specimens: Effects of parity, stage of the cycle and oral contraception

	NULLIPARAE			PAROUS WOMEN		
	On Pill	Follic phase	Luteal phase	On Pill	Follic phase	Luteal phase
Lobules/sq mm	0.291 ±0.011	0.481 ±0.140	0.582 ±0.293	0.604 ±0.168	0.347 ±0.084	0.861 ±0.242
Ductules/sq mm	5.03 ±3.19	10.18 ±6.49	7.16 ±4.16	16.67 ±12.92	6.15 ±3.37	16.33 ±5.00
% area of lobules	6.4 ±4.4	9.4 ±3.8	6.7 ±2.4	17.7 ±11.1	5.1 ±2.8	13.1 ±4.0
Average number of ductules/lobule	18.2 ±8.9	16.2 ±5.7	11.7 ±1.9	23.3 ±20.8	16.0 ±5.3	18.8 ±1.6
Area of average lobule (sq mm)	0.233 ±0.127	0.239 ±0.05	0.146 ±0.045	0.263 ±0.112	0.133 ±0.043	0.156 ±0.029
Ductule density in lobules (/sq mm)	81.5 ±6.2	93.1 ±46.4	102.4 ±26.0	78.9 ±23.3	120.2 ±2.5	136.1 ±22.6
TOTAL 23	(n:2)	(n:5)	(n:5)	(n:2)	(n:3)	(n:6)

Differences between subgroups are not significant

TABLE 4:25 Assessment of lobules

Coded specimens: Effects of stage of cycle and parity

SIMPLIFIED TABLE

	All nullips	All parous	All follic phase	All luteal phase
Lobules/sq mm	0.49 ±0.13	0.67 ±0.12	0.425 ±0.092	0.738 ±0.183
Ductules/sq mm	8.05 ±3.08	13.6 ±3.5	8.61 ±4.10	12.19 ±3.47
% area of lobules	7.8 ±1.9	11.8 ±3.0	7.8 ±2.5	10.2 ±2.6
Average no of ductules/lobule	14.7 ±2.7	18.9 ±2.6	16.1 ±3.8	15.6 ±1.6
Area of average lobule (sq mm)	0.199 ±0.033	0.169 ±0.027	0.199 ±0.039	0.152 ±0.024
Ductule density in lobules (/sq mm)	95.1 ±20.8	121.4 ±13.9	103.8 ±28.2	120.8 ±17.0
	(n:12)	(n:11)	(n_8)	(n:11)
	TOTAL	23	TOTAL	19

Differences between subgroups are not significant

c) Other factors

i) Menstrual age (Table 4-26)

The "Menstrual age" is the patient's age minus her age at menarche. Patients were divided into four groups as shown in Table 4:26, each group covering ten years of menstrual age.

Although there is variation between groups regarding some of the parameters studied, no general trend is seen. The numbers of lobules/sq mm and ductules/sq mm are smaller in the youngest age group than in the others, and this difference is significant ($P<0.05$).

ii) Birth interval (Table 4:27)

"Birth interval" is defined here as the age at first full-term pregnancy minus the age at menarche. Obviously, therefore, it applies to parous women only.

Patients were divided into five groups as shown in Table 4:28, with each group covering a five-year birth interval.

If lobule development continued before first pregnancy but was arrested after first pregnancy, a trend would be seen in Table 4:27, but no such trend is present. The numbers of specimens in some of the groups are small, but in spite of this the results are fairly constant, and differences are not significant.

iii) Breast-feeding history among parous women (Table 4:28)

Parous women in whom the history of breast-feeding was known were divided into five groups according to whether or not they had breast-fed, and for how long. The groupings according to duration of breast-feeding were chosen simply in order to provide approximately equal numbers of women in each group. The results, summarised in Table 4:28, show no trends and no significant differences, either according to length of breast-feeding, or between those who breast-fed and those who did not.

TABLE 4:26 Assessment of lobules

Effect of menstrual age

	Menstrual age (years)			
	1 - 9	10-19	20-29	30+
Lobules/sq mm	0.27 ±0.07 (n:21)	0.49 ±0.06 (n:37)	0.33 ±0.04 (n:44)	0.42 ±0.07 (n:29)
Ductules/sq mm	4.3 ±1.0 (n:17)	10.6 ±2.0 (n:29)	7.7 ±1.2 (n:37)	6.9 ±1.6 (n:18)
% area of lobules	7.06 ±1.61 (n:17)	8.57 ±1.04 (n:28)	7.45 ±1.21 (n:31)	8.70 ±1.27 (n:20)
Average no of ductules/lobules	20.7 ±2.9 (n:15)	23.0 ±1.8 (n:29)	20.1 ±1.7 (n:36)	19.3 ±2.4 (n:18)
Area of average lobule (sq mm)	0.194 ±0.028 (n:13)	0.263 ±0.039 (n:28)	0.192 ±0.030 (n:26)	0.236 ±0.051 (n:19)
Ductule density in lobules (/sq mm)	165 ±51 (n:11)	147 ±25 (n:22)	147 ±23 (n:23)	168 ±40 (n:11)

Differences between the youngest age group and the others are significant, for lobules/sq mm and ductules/sq mm ($P<0.05$). Otherwise differences are not significant.

TABLE 4:27 Assessment of lobules

Effect of birth interval among parous women

	Birth interval (years)				
	2 - 5	6 - 10	11 - 15	16 - 20	21+
Lobules/sq mm	0.64 ±0.22 (n:6)	0.36 ±0.04 (n:34)	0.45 ±0.06 (n:35)	0.45 ±0.11 (n:9)	0.83 ±0.82 (n:2)
Ductules/sq mm	3.5 ±0.1 (n:2)	7.6 ±1.2 (n:26)	10.9 ±2.0 (n:29)	7.0 ±2.1 (n:7)	9.9 ±9.9 (n:2)
% area of lobules	11.36 ±3.78 (n:5)	6.33 ±1.03 (n:20)	7.91 ±0.89 (n:27)	7.60 ±1.70 (n:6)	5.72 (n:1)
Average no of ductules/lobule	23.7 ±9.7 (n:2)	20.7 ±1.8 (n:24)	22.7 ±1.9 (n:31)	19.8 ±3.1 (n:7)	12 (n:1)
Area of average lobule (sq mm)	0.208 ±0.064 (n:5)	0.206 ±0.042 (n:20)	0.269 ±0.045 (n:27)	0.173 ±0.048 (n:7)	0.035 (n:1)
Ductule density in lobules (/sq mm)	303 ±257 (n:2)	171 ±24 (n:15)	158 ±28 (n:22)	166 ±58 (n:5)	343 (n:1)

"Birth interval": age at first full-term deliver minus age at menarche.

There are no significant differences between the groups.

TABLE 4:28 Assessment of lobules

Effect of breast-feeding history among parous women

	Length of breast-feeding					
	Never breast-fed	1-6 weeks	8-20 weeks	24-26 weeks	52-84 weeks	All breast-feeders
Lobules/sq mm	0.44 ±0.06 (n:29)	0.34 ±0.06 (n:13)	0.35 ±0.13 (n:12)	0.48 ±0.10 (n:16)	0.49 ±0.09 (n:8)	0.41 ±0.05 (n:49)
Ductules/sq mm	8.4 ±1.5 (n:22)	6.6 ±1.8 (n:7)	6.0 ±1.9 (n:11)	10.8 ±2.8 (n:13)	7.6 ±2.8 (n:5)	8.1 ±1.3 (n:36)
% area of lobules	8.47 ±1.28 (n:19)	5.77 ±1.40 (n:10)	7.77 ±2.95 (n:6)	7.05 ±0.76 (n:10)	10.54 ±2.30 (n:5)	7.34 ±0.85 (n:31)
Average no of ductules/lobule	22.2 ±1.9 (n:23)	24.0 ±4.4 (n:7)	18.9 ±3.0 (n:10)	23.8 ±2.5 (n:12)	17.5 ±3.0 (n:5)	21.5 ±1.6 (n:34)
Area of average lobule (sq mm)	0.262 ±0.046 (n:20)	0.201 ±0.041 (n:10)	0.314 ±0.146 (n:6)	0.215 ±0.666 (n:10)	0.205 ±0.046 (n:5)	0.228 ±0.037 (n:31)
Ductule density in lobules (/sq mm)	157 ±36 (n:15)	148 ±47 (n:6)	129 ±57 (n:5)	165 ±31 (n:10)	184 ±117 (n:2)	154 ±22 (n:23)

There are no significant differences between the groups

iv) Diagnosis of the lump biopsied (Table 4:29)

Patients were divided as described in Section IID of this chapter (Table 4:15) into those whose primary pathological condition was likely to be localised (or was non-existent) and those whose primary pathological condition might have been generalised or patchily distributed throughout the breast.

Only those parameters which had shown a significant variation in relation to parity were studied.

The difference between the two groups were not significant, although for each parameter (lobules/sq mm; ductules/sq mm; and ductule density within the lobules) the figures were higher in Group 2 - ie. the patients whose disease might have been widespread

v) Influence of the site of biopsy (Table 4:30)

A possible effect of the site of the biopsy was mentioned earlier (Section 2a(iii)). Patients were divided into groups according to the disease of the biopsy site from the nipple, and also according to the quadrant of the breast from which the biopsy was taken. Only the numbers of lobules per sq mm and the numbers of ductules per sq mm were studied.

Table 4:30 shows that there were no significant differences between the groups. In particular, this study does not confirm the trend shown in Table 4:20, in which greater numbers of lobules were present in the upper/outer quadrant of the breast. (If anything, the trend in Fig 4:30 is in the opposite direction).


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TABLE 4:29 Lobules: Influence of diagnosis of primary condition

	Group 1 (localised disease)	Group 2 (possibly generalised disease)
Ductules/sq mm	7.85 ±1.19 (n=52)	8.05 ±1.09 (n=45)
Lobules/sq mm	0.348 ±0.040 (n=61)	0.437 ±0.044 (n=63)
Ductule density in lobules (/sq mm)	143±22 (n=34)	169±21 (n=29)

Differences between the groups are not significant

TABLE 4:30 Lobules: Influence of site of biopsy

a) Distance from nipple (cm)	1	2	3	4	5	6 or more
Lobules/sq mm	0.41 ±0.08 (n:8)	0.46 ±0.07 (n:18)	0.51 ±0.14 (n:11)	0.44 ±0.11 (n:13)	0.33 ±0.06 (n:13)	0.37 ±0.08 (n:24)
Ductules/sq mm	7.92 ±2.08 (n:6)	8.34 ±1.58 (n:15)	8.30 ±2.63 (n:7)	10.62 ±4.11 (n:11)	6.62 ±1.94 (n:10)	7.39 ±1.53 (n:20)

b) Quadrant	Upper/outer	Upper/inner	Lower/outer	Lower/inner
Lobules/sq mm	0.369 ±0.043 (n:42)	0.439 ±0.082 (n:25)	0.429 ±0.125 (n:8)	0.683 ±0.160 (n:12)
Ductules/sq mm	8.197 ±1.169 (n:34)	7.483 ±2.525 (n:18)	9.063 ±2.091 (n:8)	11.71 ±2.498 (n:10)

Differences between the groups are not significant

C Examination of the ductules

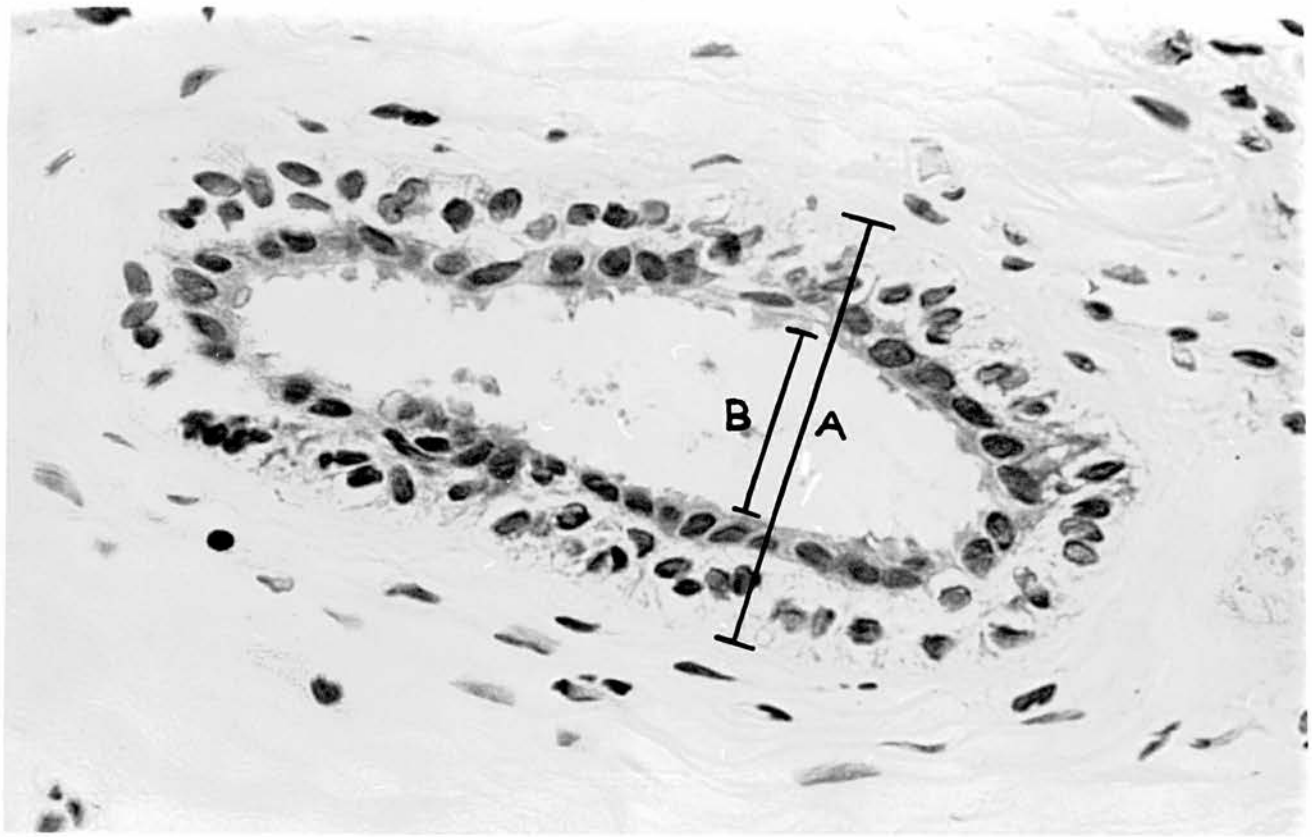
1 Methods

Examination of the ductules involved only the ductules within the lobules, and no assessment of large extralobular ductules was made, as they were seen in only a few of the sections. The examination concentrated on three areas. First, the size of the ductules, and the size of their lumina. Second, the type of cells present in the epithelium of the ductules. Third, the presence or absence of secretion in the lumen.

a) Ductule size Preliminary examination showed that there is great variation between specimens in the dilatation of the lumina of the ductules. This was assessed in two ways. First, a subjective assessment allowed rapid assessment of a large number of specimens. Second, direct measurement, a more time-consuming procedure, gave detailed information on a smaller number of specimens.

(i) Assessment of ductule dilatation - Sections were assessed subjectively according to whether the majority of ductule lumina were "very small", "small", "moderate", "dilated", or "very dilated". Two intermediate categories, "moderate/small" and "moderate/dilated", were also necessary, because ductule dilatation was rarely completely uniform over a section. The number of categories was therefore seven. The number of specimens examined by this method was 121.

(ii) Measurement of ductule diameters - Using the measuring eyepiece and the medium high-power objective lens, the diameter of the ductules could be measured directly. In each measurement, the outer diameter was measured and then the diameter of the lumen (as shown in Fig 4:5), giving the height of the epithelium when the difference between



x 800

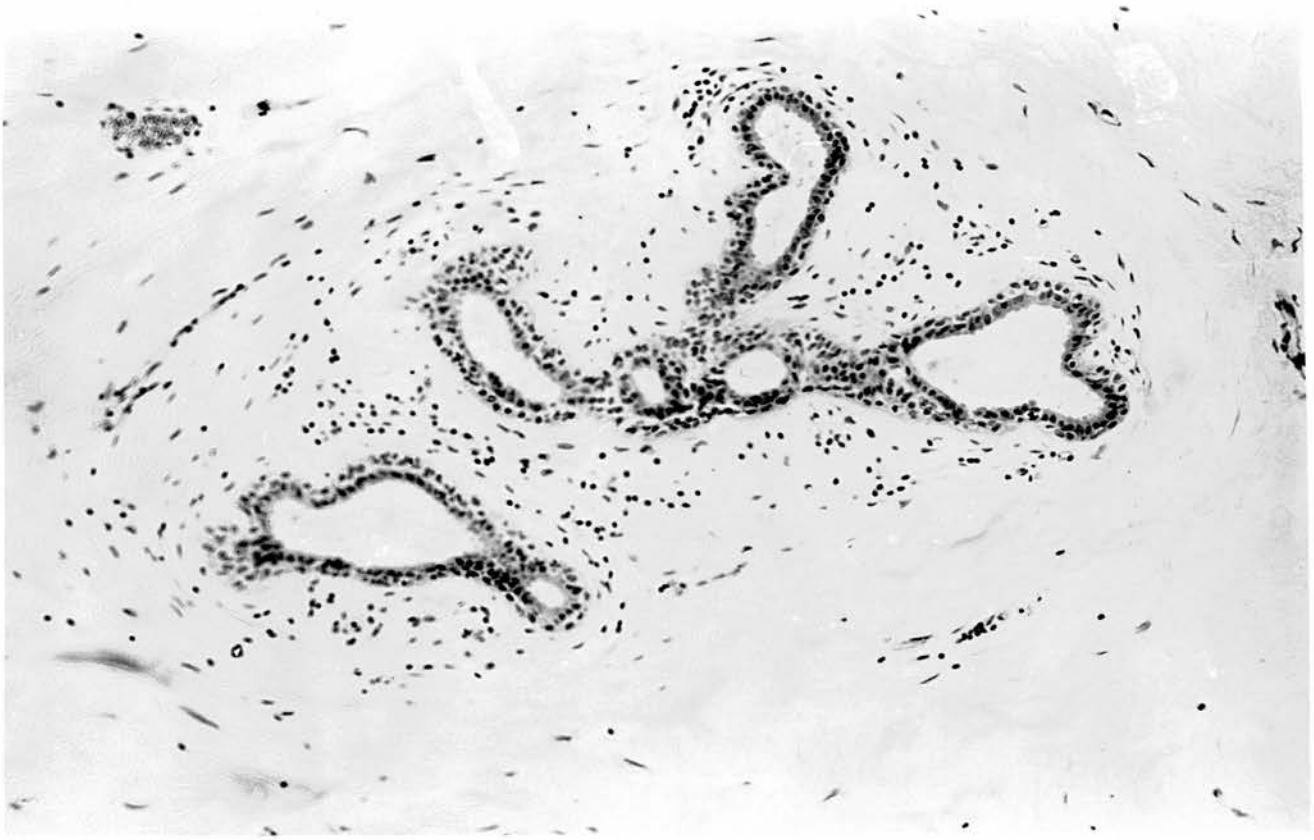
Fig 4:5 To illustrate the measurement of ductule diameters
The shortest diameter of the ductule was measured, using the measuring eyepiece.
Total ductule diameter: This measurement ("A" above) was from one basement membrane to the basement membrane on the opposite side.
Diameter of lumen: Along the same line as the above measurement, the distance between the inner borders of the epithelia was measured ("B" above).
Epithelial height: The difference between these two measurements ("A" - "B"), divided by two, gave the epithelial height.
This figure illustrates "Type 1" epithelium. The specimen was obtained from a 20 year-old woman in the seventh week of her first pregnancy. Further patient data are given at Fig 4:6

these two measurements was divided by two. To avoid artefacts due to ductules being cut at an angle, the smallest diameter of the ductule was measured. Usually 25 ductules were measured, and always at least 20. The measurement was made in arbitrary units on the eyepiece scale, and when the average diameter for each specimen was found in these units, the figure was converted to microns (μ) - the conversion factor having been found using a measuring slide. (One unit on the measuring eyepiece was equivalent to 23.5μ). A total of 47 sections was examined by this method.

b) Ductule epithelium After examination of a number of specimens, it became clear that there were two types of cell in the epithelium of the ductules. These were cells whose cytoplasm remained clear on staining with haematoxylin and eosin, and cells with pink cytoplasm. The cells with pink cytoplasm are usually situated near the lumen of the ductule, while the clear cells are basal in position. (The clear cells are often thought to be myoepithelial cells (Hamperl 1970), but are sometimes present in larger numbers than other epithelial cells (Haagensen 1971) - a fact which casts doubt on their role as myoepithelial cells (Bassler 1970).) Both the numbers of the two types of cell, and the size of the cells, were examined.

(i) Epithelial types - The relative numbers of the two types of cell were used to grade the epithelium into different types, depending on which cell type was predominant. Initially a division into five epithelial types was attempted, but since observer agreement on five types was very difficult to achieve, a simpler classification into three types was used.

Type 1: The epithelium is two-layered, with an inner layer of "pink cells" and a complete (or almost complete) outer layer of "clear cells". (Fig 4:5 and Fig 4:6).



x 200

Fig 4:6: To illustrate "Type 1" epithelium
(See also high-power view at Fig 4:4)
The specimen was obtained at biopsy (diagnosis of lump: fibroadenoma) from a 20 year-old women seven weeks after her last menstrual period. She had had a positive pregnancy test and the pregnancy was terminated for social reasons under the same anaesthetic while the breast biopsy was done. The plasma progesterone concentration was "over 21.08 ng/ml". She had never been pregnant before. Her menstrual cycle was usually regular, 5/35 days. She had never been on the Pill. Her menarche had been at age 14.

Type 2: The two cell types are present but are intermingled and do not form discrete layers. (Fig 4:7 and Fig 4:8).

Type 3: Only "pink cells" are present, and there are no "clear cells". (Fig 4:9 and Fig 4:10).

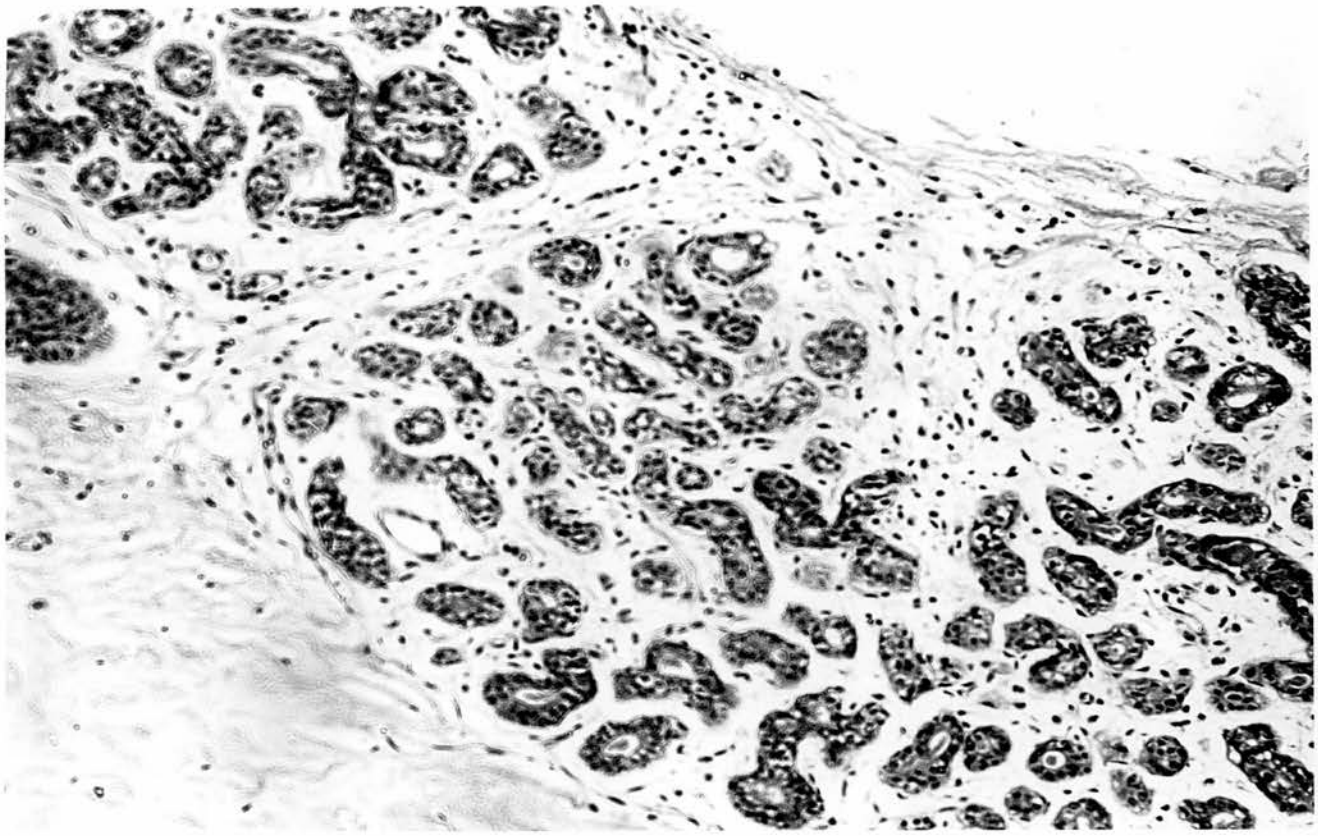
(ii) Size of epithelial cells - Separate assessments were made of the size of the "pink cells" and the "clear cells". Each specimen was graded into one of three types depending on whether the pink cells were "large", "medium" or "small", and a separate grading was carried out based on the clear cells, which were divided into similar categories. Because of variation between ductules and lobules, such gradings are only approximate, but no intermediate gradings were used.

c) Secretion in lumina of ductules Secretion appears as pink-staining material in the lumina of some ductules on haematoxylin and eosin stained sections. Sections were graded as "-" when none was present; "±" when secretion was present in only a few ductules; "+" when secretion was present in some ductules in several lobules; "++" when secretion was present in most ductules in many lobules; and "+++" when secretion was present in all, or almost all, ductules.

2 Results

a) Assessment of ductule dilatation

(i) Variation (Table 4:31) - In five cases two specimens from the same breast were compared, and in three cases specimens from right and left breasts were compared. The results are summarised in Table 4:31, and show fairly good correlation between specimens. In six of the eight cases assessment was identical in the two specimens. In one case, two specimens from one breast differed by one "grade". In the remaining case, two specimens (one from each breast) differed markedly, with ductule lumina graded as "moderate/dilated" in one specimen and "small" in the other.



x 200

Fig 4:7 To illustrate "Type 2" epithelium
(See also high-power view at Fig 4:8)

The specimen was obtained at biopsy (diagnosis of lump: fibroadenosis) from a 40 year-old woman on the 25th day of a 26-day cycle. The plasma progesterone concentration was 4.55 ng/ml. Her menarche had been at age 13. She had had two full-term pregnancies, one when she was 20 years old, and the other at age 21. She had breast-fed for 10 days after the first pregnancy, but not at all after the second. She had no breast tenderness during the cycle.

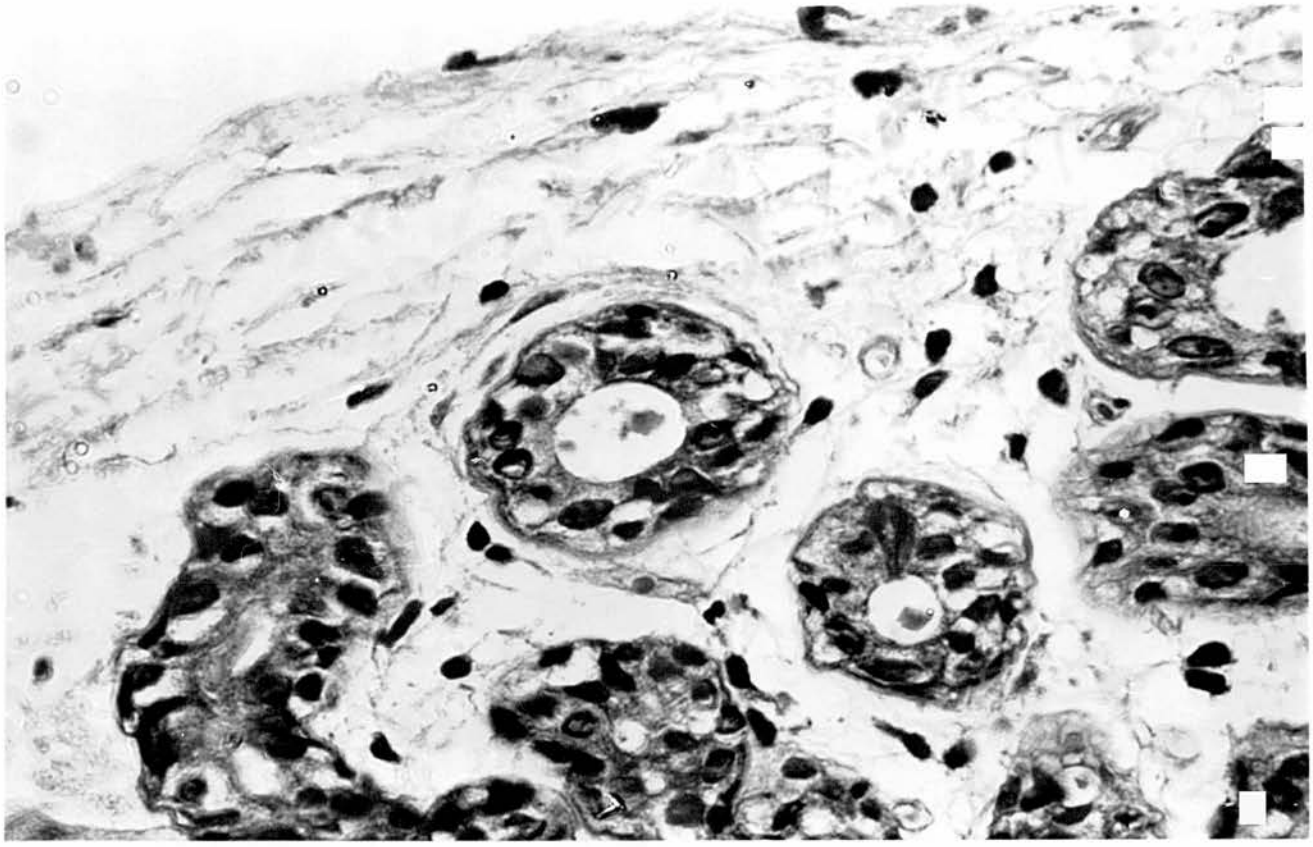


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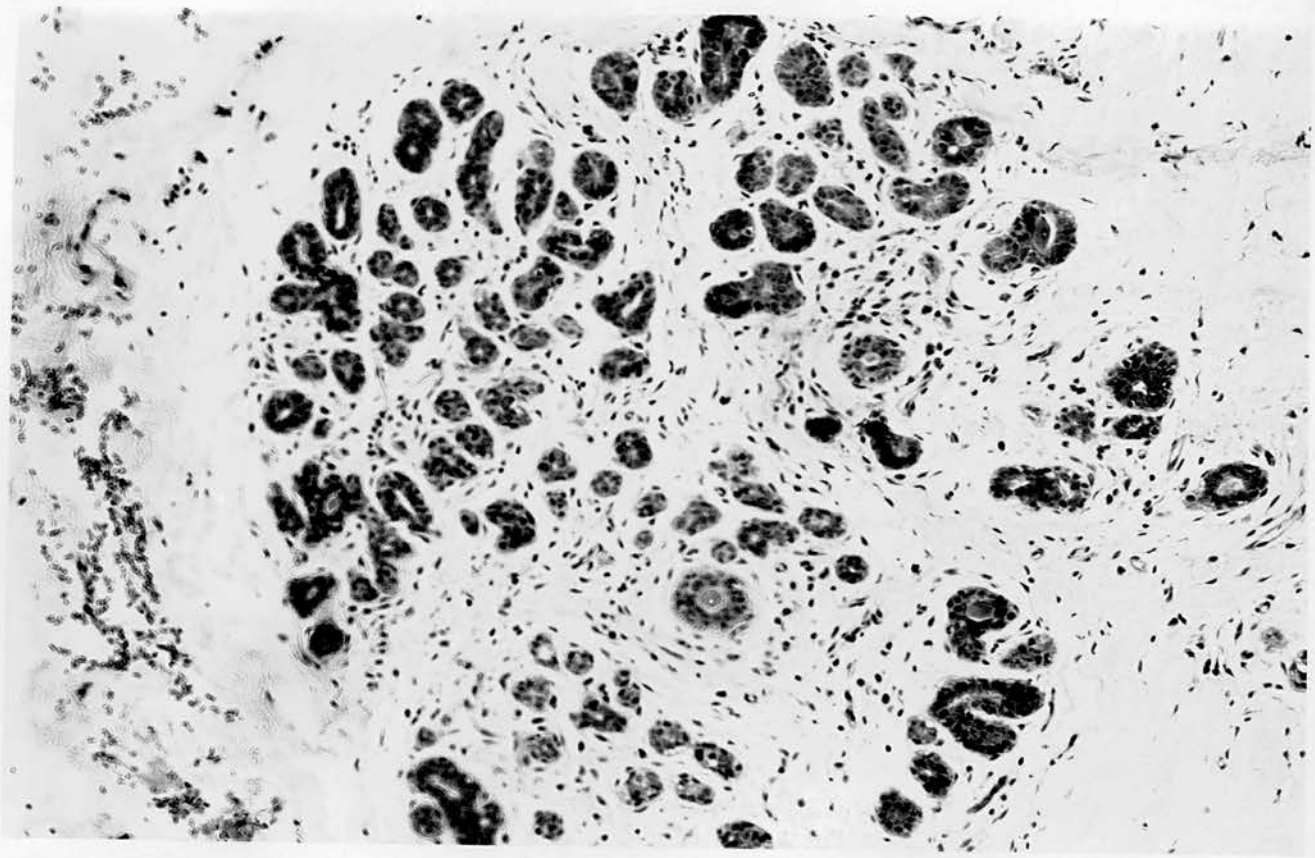
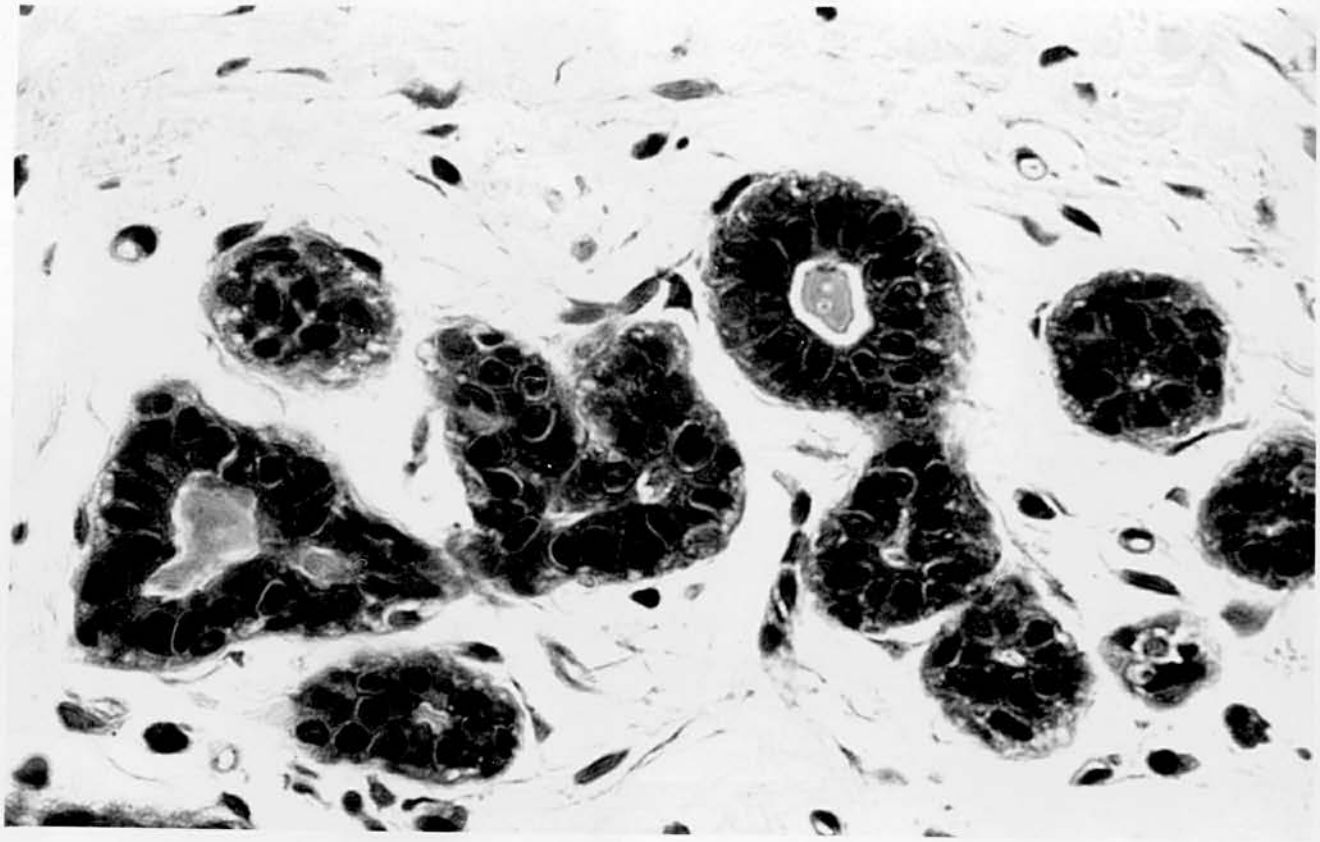
Fig 4:8 To illustrate "Type 2" epithelium
This illustration is of the same specimen seen in lower-power view in Fig 4:7. The patient data are given at Fig 4:7.



x 200

Fig 9: To illustrate "Type 3" epithelium
(See also high-power view at Fig 4:10)
The specimen was obtained at biopsy (diagnosis of lump: fibroadenosis) from a 28 year-old women on the second day of a 26-day cycle. The plasma progesterone concentration was "undetectable". Her menarche had been at age 14. She had had two miscarriages and four full-term pregnancies, the first when she was 19 years old and the most recent full-term pregnancy at age 22. She had breast-fed her first and third babies for six weeks each, but had not breast-fed the other two. She had no breast symptoms during the cycle.



x 800

Fig 4:10 To illustrate "Type 3" epithelium
This illustration is of the same specimen seen in lower-power view in Fig 4:9. The patient data are given at Fig 4:9.

TABLE 4:31 Assessment of ductule lumina
Variation between specimens from the same patient

Patient data	Right breast		Left breast	
	Specimen A	Specimen B	Specimen A	Specimen B
Parous, follic phase	Mod/dilated		Small	
Parous, follic phase	Mod/small	Mod/small		
Nullip, follic phase			Small	Small
Nullip, on Pill	Moderate		Moderate	
Nullip, on Pill	Small	Small		
Nullip, luteal phase	Small	Small		
Nullip, luteal phase			Mod/dilated	Moderate
Nullip, luteal phase	Moderate		Moderate	

(ii) Effects of parity and the cycle (Table 4:32) - 121 specimens were divided into groups first according to whether the woman was parous or nulliparous, and then according to whether she was taking oral contraceptives or was in the proliferative or luteal phase of a normal cycle. (The luteal phase was defined as a plasma progesterone concentration of over 1 ng/ml). The results are presented in Table 4:32.

TABLE 4:32 Assessment of ductule lumina
Effects of parity, oral contraception and the menstrual cycle

DUCTULE LUMINA (and total numbers of patients)	NULLIPARAE (n:40)			PAROUS WOMEN (n:81)		
	On Pill (n:9)	Follic phase (n:13)	Luteal phase (n:18)	On Pill (n:9)	Follic phase (n:41)	Luteal phase (n:31)
Very small (1)					1	
Small (48)	3	8	7	3	12	15
Mod/small (5)					3	2
Moderate (43)	4	4	4	5	17	9
Mod/dilated (8)	1	1	2		2	2
Dilated (13)	1		4	1	4	3
Very dilated (3)			1		2	

Total number of specimens examined: 121

NOTE: Among nulliparous patients there is a trend to greater numbers of dilated ductules in the luteal phase compared with the follicular phase. Its significance was tested as follows:-

	Follicular phase	Luteal phase
"Small" (including moderate/small and "very small)	8	7
"Moderate"	4	4
"Dilated" (including moderate/dilated and "very dilated")	1	7

Chi-square applied to these figures = 3.86 (not significant)

Table 4:32 shows no effect of parity. When the results for nulliparae are totalled and compared with those for parous women the pattern is identical. There is also no effect from oral contraception. Among parous women the pattern is similar in the two phases of the cycle , but among nulliparae there are more "dilated" lumina during the luteal phase of the cycle than during the proliferative phase. This trend is not significant when the Chi-square test is applied as shown in the footnote to Table 4:32.

(iii) Other factors (Tables 4:33 4:34 4:35) -

Menstrual age The patients were divided into groups according to menstrual age, as described previously (Section Bc(i), page 4-47) and as shown in Table 4:33. There is no trend and no significant effect of menstrual age.

Birth interval When the patients were divided into groups according to birth interval, as described previously (Section Bc(ii)), no effect was seen (Table 4:34).

Breast-feeding history Table 4:35 shows no effect of breast-feeding compared with women who never breast-fed, and no effect according to the length of time for which they breast-fed.

b) Measurement of ductule diameters

(i) Variation (Tables 4:36 and 4:37) - Table 4:36 shows the variation in total ductule diameter, luminal diameter and epithelial height between ten sections from the same block of tissue. Variation is smallest with the measurement of epithelial height (coefficient of variation = 1.5) and is larger with the measurements of the diameter of the lumen (coefficient of variation = 14.2)

TABLE 4:33 Assessment of ductule lumina
Effect of menstrual age

	MENSTRUAL AGE			
	1 - 9 (n:19)	10 - 19 (n:32)	20 - 29 (n:41)	30+ (n:25)
Very small (1)		1		
Small (45)	10	11	17	7
Moderate/small (5)		1	3	1
Moderate (43)	5	15	10	13
Moderate/dilated (7)	1	1	4	1
Dilated (13)	3	2	5	3
Very dilated (3)		1	2	

Total number of specimens examined 117

In each age group the distribution is similar

TABLE 4:34 Assessment of ductule lumina
Effect of birth interval

	BIRTH INTERVAL				
	2 - 5 years (n:5)	6 - 10 years (n:28)	11 - 15 years (n:33)	16 - 20 years (n:10)	21+ years (n:1)
Very small (1)		1			
Small (29)	2	7	13	7	
Moderate/small (5)		2	2	1	
Moderate (30)	3	12	12	2	1
Moderate/dilated (3)			3		
Dilated (7)		5	2		
Very dilated (2)		1	1		

Total number of specimens examined 77

There are no significnat differences between groups of patients

TABLE 4:35 Assessment of ductule lumina
Effects of history of breast feeding among parous women

		Never breast-fed	Length of breast-feeding				All breast-feeders
			1-6 wks	8-20 wks	24-46 wks	52-84 wks	
Very small (1)					1		1
Small (26)		12	4	6	1	3	14
Moderate/small (5)		2	1		2		3
Moderate (27)		10	4	3	6	4	17
Moderate/dilated (3)		1			2		2
Dilated (7)		3	3		1		4
Very dilated (1)						1	1
Total examined	70	28	12	9	13	8	42

The groups of patients show no significant differences

TABLE 4:36 Measurement of ductule diameters:

Variation between sections from the same block

	Total Ductule Diameter (μ)	Diameter of Lumen (μ)	Epithelial Height (μ)
	259	86	86
	240	69	86
	252	81	85
	268	90	89
	237	79	79
	256	85	86
	248	67	91
	249	89	80
	226	61	82
	223	63	80
Mean	245	77	84
SD	14.3	11.0	4
SEM	4.5	3.5	1.3
Coefficient of variation	5.8	14.2	1.5

The patient was a parous woman in the luteal phase of the cycle

TABLE 4:37 Measurement of ductule diameters:

Variation between different specimens from the same patient

a) TOTAL DUCTULE DIAMETER (μ)

	Right breast		Left breast	
	Specimen A	Specimen B	Specimen A	Specimen B
Patient data				
Nullip, follic			158	134
Nullip, on Pill	230		273	
Nullip, luteal	221	212		
Nullip, on Pill	296	247	218	252
	249		266	

b) DIAMETER OF LUMEN (μ)

Patient number				
Nullip, follic			35	28
Nullip, on Pill	80		103	
Nullip, luteal	82	73		
Nullip, on Pill	120	66	54	82
	66		94	

c) EPITHELIAL HEIGHT (μ)

Patient number				
Nullip, follic			61	80
Nullip, on Pill	75		85	
Nullip, luteal	70	70		
Nullip, on Pill	88	90	82	85
	92		86	

Table 4:37 shows the variation between specimens in each of the parameters, in four cases. Two of the cases each had two biopsies from one breast, one case had bilateral biopsies, and the fourth had three samples taken from each breast at mammoplasty. Epithelial height shows little variation within one breast, but slightly more variation between right and left breasts. The diameter of the lumen shows greater variation, both in a single breast and between right and left breasts.

(ii) Effect of parity and the menstrual cycle (Table 4:38) - As before, the patients were divided into groups according to parity, use of oral contraception and stage of the cycle. The results are shown in Table 4:38. There is no overall difference between parous and nulliparous women. As in section 2a(ii), there is a trend among nulliparous women to have greater ductule luminal diameters during the luteal phase of the cycle, but this trend does not reach statistical significance. No such trend is seen among parous women.

Epithelial height, though showing no difference between nulliparae and parous women, shows variation with the cycle in both groups. The height is greater in the luteal phase of the cycle, and this difference is significant (among parous women $P<0.01$; among nulliparae $P<0.05$).

Oral contraceptive use shows no effect on luminal diameter, but epithelial height is greater among nulliparous users of oral contraceptives than among normally cycling nulliparae ($P<0.05$). No such effect of oral contraceptive use is seen among parous women.

TABLE 4:38 Measurement of ductule diameters:

Effects of parity, stage of cycle and oral contraception

		NULLIPARAE			PAROUS WOMEN		
		On Pill	Prolif Phase	Luteal Phase	On Pill	Prolif Phase	Luteal Phase
a)	TOTAL DUCTULE DIAMETER (μ)	248 ±8	172 ±12	247 ±31	188 ±38	178 ±9	209 ±14
	Total: 47	(n:10)	(n:5)	(n:3)	(n:2)	(n:12)	(n:15)
b)	DIAMETER OF LUMEN (μ)	83 ±7	52 ±12	101 ±24	66 ±28	55 ±6	59 ±7
	Total: 42	(n:9)	(n:4)	(n:3)	(n:2)	(n:12)	(n:12)
			NS				
c)	EPITHELIAL HEIGHT (μ)	84 ± 2 *	60 ± 2 *	73 ± 3	61 ± 5	62 ± 3 *	74 ± 5
	Total: 42	(n:9)	(n:4)	(n:3)	(n:2)	(n:12)	(n:12)

Differences in epithelial height between nulliparous women in the proliferative phase and the other two groups of nulliparous women are significant ($P<0.05$). The difference in epithelial height between the proliferative phase and the luteal phase among parous women is significant ($P<0.01$). Other differences are not significant.

iii) Other factors (Tables 4:39 4:40 4:41) - The effect of menstrual age is shown in Table 4:39. There is a trend towards decreasing luminal diameter with greater age, and this trend is statistically significant ($P<0.05$). There is also a slight trend towards decreasing epithelial height with greater age, but this trend is not significant.

The effect of birth interval is shown in Table 4:40. Because of low sample numbers some groups have one or no results, and the trend (towards lower figures with increasing birth interval) is not significant.

The effect of breast-feeding history is shown in Table 4:41. Although patients who had breast-fed for more than a year in total had greater luminal diameters and epithelial heights than other groups, the numbers are again small and the difference is not significant. There is no overall difference between women who breast-fed and those who did not as regards epithelial height. There is a slight difference as regards luminal diameters, with those who had breast-fed having greater diameters than those who did not, but this trend is not statistically significant.

4:39 Measurement of ductule diameters:

Effects of menstrual age

	MENSTRUAL AGE			
	1 - 9 years	10 - 19 years	20 - 29 years	30+ years
a) TOTAL DUCTULE DIAMETER (μ)	253 ±24	229 ±10	194 ±12	172 ±13
Total: 43	(n:4)	(n:19)	(n:12)	(n:8)
b) DIAMETER OF LUMEN (μ)	101 ±17	74 ±5	62 ±8	45 ±5
Total: 38	(n:4)	(n:19)	(n:9)	(n:6)
c) EPITHELIAL HEIGHT (μ)	76 ±5	79 ±3	61 ±4	62 ±7
Total: 38	(n:4)	(n:19)	(n:9)	(n:6)

The trend towards decreasing luminal diameter with increasing menstrual age is significant ($P<0.05$).

TABLE 4:40 Measurement of ductule diameters:

Effects of birth interval among parous women

	BIRTH INTERVAL			
	2 - 5 years	6 - 10 years	11 - 15 years	16 - 20 years
a) TOTAL DUCTULE DIAMETER (μ)	174	218 ±14	177 ±11	195
Total: 27	(n:1)	(n:1)	(n:13)	(n:1)
b) LUMEN DIAMETER (μ)	54	67 ±8	50 ±6	
Total: 24	(n:1)	(n:11)	(n12)	
c) EPITHELIAL HEIGHT (μ)	60	75 ±5	62 ±4	
Total: 24	(n:1)	(n:11)	(n:12)	

Differences between groups are not significant

TABLE 4:41 Measurement of ductule diameter:

Effects of breast-feeding history among parous women

		Length of breast-feeding					
		Never breast-fed	1 - 6 weeks	8 - 20 weeks	24 - 46 weeks	52 - 84 weeks	All breast-feeders
a)	TOTAL DUCTULE DIAMETER (μ)	187 ±15	191 ±14	149 ±13	183 ±40	252 ±5	197 ±11
	Total 28	(n:7)	(n:6)	(n:5)	(n:4)	(n:6)	(n:21)
b)	LUMEN DIAMETER (μ)	48 ±5	51 ±16	43 ±6	61 ±15	82 ±3	61 ±6
	Total 23	(n:5)	(n:4)	(n:5)	(n:4)	(n:5)	(n:18)
c)	EPITHELIAL HEIGHT (μ)	66 ±6	66 ±9	53 ±4	62 ±7	85 ±1	68 ±4
	Total 23	(n:5)	(n:4)	(n:5)	(n:4)	(n:5)	(n:18)

Differences between groups are not significant

c) Epithelial types

(i) Variation - Observer variation: 23 specimens were assessed independently by two observers (TJA and myself). The specimens had been selected to represent different groups of patients, and had been relabelled to ensure that the assessment was "blind". The results are shown in Table 4:42.

This table shows complete agreement between observers in only four cases. In another 14 cases there was agreement about the predominant type of epithelium present, but disagreement over the amount of other type also present. In the remaining five cases (numbered h, m, n, o and s) there was disagreement about the predominant type of epithelium. In these five cases the disagreement was limited to a single grade - ie. a specimen assessed as "Type 2" by one observer was assessed as "Type 3(2)" by the other, for example. In no case was an epithelium assessed as Type 1 by one observer and Type 3 by the other.

Variation between specimens from the same patient: In five cases two specimens were available from the same breast, and in another six cases bilateral biopsies were available. Three more patients had two or more specimens taken from each breast at mammoplasty. The assessment of these 14 patients is shown in Table 4:43, along with data on the patients.

In seven cases there was complete agreement or almost complete agreement ("almost complete" meaning that there was agreement about the types present but slight disagreement about their proportions - ie. 2>3 as compared with 2≫3).

In two of the remaining seven cases there was agreement on the predominant type of epithelium present but another type was also present in only one of the pair of specimens. In the remaining five cases there was agreement on which types of epithelium were present, but disagreement over which type was predominant.

In no case did the disagreement involve more than one grade - ie. no patient had Type 1 in one specimen and Type 3 in another (apart from the single section in which types 1 and 3 were found together).

Agreement tended to be slightly better between pairs of specimens taken from the same breast (as compared with bilateral biopsies, but the difference was very slight.

(ii) Effect of parity and the menstrual cycle - The patients were divided into groups according to parity and according to oral contraceptive use and stage of the cycle. The detailed results are presented in Table 4:44, with a simplified version of the table on the same page.

Parity: When the overall pattern in nulliparae is compared with that in parous women, it is seen that there is a predominance of Type 1 epithelium among nulliparae, while Type 3 epithelium is relatively more common among parous women. In the simplified table, "Type 1 present" means that type 1 epithelium was in some cases not the predominant type present, and in most cases was mixed with Type 2 epithelium in varying proportions. The same applies to "Type 3 present" in the simplified table. The effects of parity are presented in graph form in Fig 4:11. The difference between parous women and nulliparae is highly significant (Chi square 38.47; $p<0.001$).

TABLE 4:42 Epithelial types: Observer variation

		Observer 1 (JOD)	Observer 2 (TJA)
Nulliparae			
Follicular phase	(a)	1 & 2	2 > 3 > 1
	(b)	2	2
	(c)	3 (2)	3 > 2
	(d)	1 & 2 (3)	2 > 3
	(e)	2	1 & 2
Luteal phase	(f)	1 & 2 & 3	3 > 2
	(g)	2 (1)	2
	(h)	2	3 > 2
	(i)	2	1 & 2
	(j)	1 & 2 & 3	3
On Pill	(k)	3	2 & 3
	(l)	2	2 > 3
Parous			
Follicular phase	(m)	2	3 > 2
	(n)	2	3 > 2
	(o)	1 (2)	2 > 1 3
Luteal phase	(p)	2	2 > 3
	(q)	2	3 & 2
	(r)	2 & 3	3
	(s)	1 (2)	2
	(t)	2	2
	(u)	2 & 3	3
On Pill	(v)	1 (2)	1 > 2
	(w)	2	2 > 3 > 1

"&" means the types were present in equal numbers

( ) means that only a small proportion of lobules had this type.

TABLE 4:43 Epithelial types:

Variation between specimens from the same patient

PATIENT DATA	Right breast		Left breast	
	Specimen A	Specimen B	Specimen A	Specimen B
Parous, follic phase	2		2	
Parous, follic phase	2>>3	2>3		
Nullip, follic phase			1>2	2
Nullip, on Pill	1>2		1>2	
Nullip, luteal phase	1>2	1>2		
Nullip, luteal phase	2>>1	2>>1		
Nullip, luteal phase			2>>1	2>1
Nullip, luteal phase	1>2		2>1	
Nullip, on Pill	2		2>3	
Nullip, follic phase	1>2	2>1	2>>1 2>>1	2>>1
Nullip, follic phase	3>2		2	
Nullip, on Pill	2>>1 1 & 2 & 3	1>2	2>>1 1>2	2>1
Nullip, follic phase	2		2>3	
Nullip, follic phase	2>>1	2>>1	2>>1	2

TABLE 4:44 Epithelial types: Effects of parity and menstrual cycle

Epithelial type (and number of patients)	Nulliparae			Parous		
	On Pill	Prolif phase	Luteal phase	On Pill	Prolif phase	Luteal phase
1 only (3)		1	1		1	
1≫2 (6)	1	1	2			2
1>2 (17)	4	4	5	1	2	1
2>1 (15)	3	4	3		5	
2≫1 (32)	2	10	6	1	10	3
2 only (45)	2	7	2	7	17	10
2≫3 (6)		1		1	3	1
2>3 (15)	1	2	1	2	2	7
3>2 (8)					5	3
3≫2 (10)				1	2	7
3 only (17)	1	2		3	7	4
3 & 1 (2)		1			1	
1&2&3 (2)	1		1			
Total 178	15	33	21	16	55	38

SIMPLIFIED TABLE:

Type 1 present (73)	10	20	17(47)	2	18	6(26)
Type 2 only (45)	2	7	2(11)	7	17	10(34)
Type 3 present (56)	2	5	1(8)	7	19	22(48)
Other	1	1	1		1	
	15	33	21	16	55	38

The overall difference between nulliparous and parous women is highly significant ($x^2 = 38.47$; $P<0.001$)

Other differences are not significant

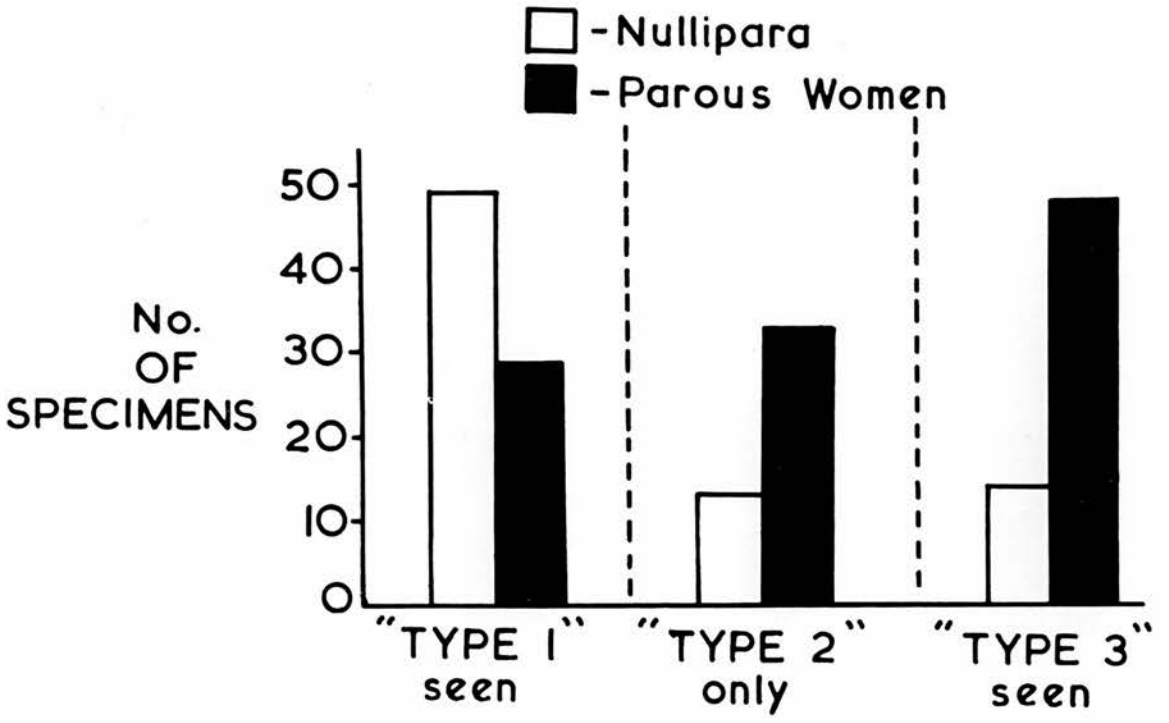


Fig 4:11 Epithelial types: distribution among parous and nulliparous women.
The histogram shows the frequency with which the three types of epithelium shown in Figs 4:5 to 4:10 were seen in 178 biopsies of normal breast tissue. Type 1 epithelium was seen mainly in nulliparous women, and Type 3 epithelium was commoner in parous women. Compare Table 4:44

Oral contraception and the menstrual cycle: Table 4:44 shows that the pattern among oral contraceptive users was very similar to that among normally cycling women. In both parous and nulliparous women there was no difference between the follicular and luteal phases of the cycle.

Coded specimens: The distribution of epithelial types among the coded specimens was also checked, in an attempt to eliminate observer bias. The code was not broken until the assessments had been made. The results are shown below (Table 4:45)

TABLE 4:45 Frequency of predominant epithelial type among coded specimens

Predominant type	Nulliparae		Parous women	
	Follic phase	Luteal phase	Follic phase	Luteal phase
Type 1	2	2	1	1
Type 2	4	5	2	5
Type 3	1	2	0	2

Total number of specimens examined = 23 (in four specimens two epithelial types were equally distributed).

From the small numbers no trend is apparent, and this small series neither confirms nor refutes the trend in the previous table.

(iii) Other factors (Tables 4:46 to 4:52) - Menstrual age: Patients were divided into four groups as before, each group covering a ten-year menstrual age range. The results are presented in Table 4:46. The simplified table at the bottom of the page shows a trend, with the youngest age range having a greater frequency of Type 1 epithelium.

In order to separate this effect from the effect of parity, the effect

TABLE 4:46 Epithelial types: Effects of menstrual age

		Menstrual age group			
		1 - 9	10 - 19	20 - 29	30+
1 only	(3)	2			1
1>>2	(6)	3	1	2	
1>2	(16)	3	7	5	1
2>1	(15)	4	3	7	1
2>>1	(30)	10	12	2	6
2 only	(43)	4	12	18	9
2>>3	(6)	1	1		4
2>3	(15)	3	4	4	4
3>2	(8)		1	2	5
3>>2	(9)	1	3	4	1
3 only	(17)	2	7	4	4
3 & 1	(2)		1		1
1 & 2 & 3	(2)		2		
Total examined	172	33	54	48	37

SIMPLIFIED TABLE:

Type 1 present (70)	22	23	16	9
Type 2 only (43)	4	12	18	9
Type 3 present (55)	7	16	14	18
Other (4)		3		1
	33	54	48	37

Younger menstrual age is associated with a greater frequency of Type 1 epithelium. See separate examination of parous and nulliparous women in Tables 4:47 and 4:48

TABLE 4:47 Epithelial types: Effect of menstrual age among nulliparae

		Menstrual age group			
		1 - 9	10 - 19	20 - 29	30+
1 only	(2)	2			
1>>2	(4)	3		1	
1>2	(12)	3	3	5	1
2>1	(10)	4	3	3	
2>>1	(18)	10	7		1
2 only	(10)	3	2	2	3
2>>3	(1)	1			
2>3	(4)	3	1		
3>2	(0)				
3>>2	(0)				
3 only	(3)	1		1	1
3 & 1	(1)		1		
1&2&3	(2)		2		
Total examined	67	30	19	12	6

SIMPLIFIED TABLE:

Type 1 present (46)	22	13	9	2
Type 2 only (10)	3	2	2	3
Type 3 present (8)	5	1	1	1
Other (3)		3		
	30	19	12	6

The distribution of epithelial types is similar in each age group

TABLE 4:48 Epithelial types: Effect of menstrual age among parous women

		Menstrual age group			
		1 - 9	10 - 19	20 - 29	30+
1 only	(1)				1
1>>2	(2)		1	1	
1>2	(4)		4	1	
2>1	(5)			4	1
2>>1	(12)		5	2	5
2 only	(33)	1	10	16	6
2>>3	(5)		1		4
2>3	(11)		3	4	4
3>2	(8)		1	2	5
3>>2	(9)	1	3	4	1
3 only	(14)	1	7	3	3
3 & 1	(1)				1
Total examined	105	3	35	36	31

SIMPLIFIED TABLE:

Type 1 present (24)		10	7	7
Type 2 only (33)	1	10	16	6
Type 3 present (47)	2	15	13	17
Other (1)				1
	3	35	36	31

Differences in the distribution of epithelial types in the different age groups are not significant

of menstrual age was examined separately in nulliparous and parous women (Table 4:47 and 4:48). When the simplified tables for nulliparae and parous women are compared, no effect of menstrual age is seen in either group, and it appears that the apparent effect seen in Table 4:46 is indeed due to the greater numbers of nulliparae in the younger age group.

Birth interval: The patients were divided into four groups according to the length of time between the menarche and the first full-term pregnancy. The results are presented in Table 4:49. The distribution of epithelial types is similar in each group, and no trends are seen.

Breast-feeding history: The patients were divided into groups according to whether or not they had breast-fed, and the total duration of breast-feeding. The results are presented in Table 4:50. There is no obvious difference between those who had breast-fed and those who had not, and there is no obvious effect of the duration of breast-feeding.

Diagnosis of the primary condition: The patients were divided into two groups according to the diagnosis of the condition that had necessitated operation. As described in section IID, Group 1 consisted of conditions which were localised or non-existent, and Group 2 consisted of conditions which might have been generalised or patchy in distribution. The results are shown in Table 4:51. From the simplified table it is seen that type 1 epithelium was slightly commoner among Group 1 than Group 2. This trend failed to reach statistical significance, however, and is most probably due to the greater proportion of nulliparae in Group 1.

Site of biopsy: The patients were divided into groups according to the distance between the nipple and the biopsy site, and according to

TABLE 4:49

Epithelial types: effect of birth interval among parous women

		Birth interval (years)				
		2 - 5	6 - 10	11 - 15	16 - 20	21+
1 only	(1)			1		
1>>2	(2)		1	1		
1>2	(4)		3	1		
2>1	(5)		2	2		1
2>>1	(12)		4	5	2	
2 only	(33)	3	13	12	5	
2>>3	(5)		1	3	1	
2>3	(10)		5	5		
3>2	(7)	2	1	3	1	
3>>2	(9)		6	2	1	
3 only	(13)	4	5	4		
3 & 1	(1)			1		
Total examined	102	10	41	40	10	1

SIMPLIFIED TABLE:

Type 1 present (24)	1	10	10	2	1
Type 2 only (33)	3	13	12	5	
Type 3 present (44)	6	18	17	3	
Other (1)			1		
	10	41	40	10	1

Distribution is similar in each group

TABLE 4:50

Epithelial types: effects of breast feeding history among parous women

		Breast feeding history				
		Did not breast feed	1-6 wks	8-20 wks	24-46 wks	52-84 wks
1 only	(1)		1			
1>>2	(2)	2				
1>2	(4)	1	2			1
2>1	(5)	3	2			
2>>1	(13)	6		1	5	1
2 only	(30)	13	4	5	6	2
2>>3	(4)	2	1		1	
2>3	(10)	3	1	2	3	1
3>2	(6)	1	1	2	1	1
3>>2	(6)	1	2		1	2
3 only	(15)	9	2	3	1	
3 & 1	(1)				1	
Total examined	97	41	16	13	19	8

SIMPLIFIED TABLE:						All breast feeders
Type 1 present	(25)	12	5	1	5	2	13
Type 2 only	(30)	13	4	5	6	2	17
Type 3 present	(41)	16	7	7	7	4	25
Other	(1)				1		1
		41	16	13	19	8	56

Distribution is similar in each group of patients

TABLE 4:51 Epithelial types and diagnosis of primary condition

		Group 1 (localised disease)	Group 2 (possibly generalised disease)
1 only	(3)	2	1
1>>2	(6)	5	1
1>2	(17)	9	8
2>1	(15)	7	8
2>>1	(32)	19	13
2 only	(45)	17	28
2>>3	(6)	4	2
2>3	(15)	7	8
3>2	(8)	3	5
3>>2	(10)	3	7
3 only	(17)	7	10
3 & 1	(2)	2	0
1&2&3	(2)	1	1
Total	178	86	92

SIMPLIFIED TABLE:

Type 1 present (73)	42	31
Type 2 only (45)	17	28
Type 3 present (56)	24	32

The difference between the two groups is not significant (X^2:5.13)

TABLE 4:52 Epithelial types: Effect of site of biopsy

a)

Distance from nipple (cm)	1	2	3	4	5	6 or more
Type 1 seen	3	4	4	8	9	14
Type 2 only	7	5	6	2	4	9
Type 3 seen		12	8	6	5	8
TOTAL 114	10	21	18	16	18	31

b)

Quadrant of breast	upper/outer	upper/inner	lower/outer	lower/inner
Type 1 seen	24	9	4	3
Type 2 only	17	11	3	5
Type 3 seen	16	12	5	9
TOTAL 118	58	32	12	16

In neither table are the differences significant

the quadrant of the breast from which the biopsy was taken. The results are shown in Table 4:52.

From these results there appears a slight trend for Type 1 epithelium to be found in the biopsies taken further away from the nipple, and in the upper/outer quadrant of the breast. However, this was not a significant trend. It may be that this slight trend is also explained by the site of biopsy among nulliparous women.

d) Size of epithelial cells

(i) Variation (Table 4:53) - In six cases two specimens were available for comparison: four pairs each from a single breast, and two pairs from right and left breasts. The size of clear cells and pink-staining cells was estimated separately in each pair, and the results are shown in Table 4:53. Complete agreement is seen in all specimens except in the estimation of pink-staining cells in one pair from right and left breasts. In this pair the assessment varied by only one grade (medium in one specimen and large in the other).

(ii) Effects of parity and the menstrual cycle, and of oral contraceptives (Table 4:54) -

Patients were divided into groups as before according to parity, and according to oral contraceptive use and stage of the normal cycle. The results are presented in Table 4:54.

When "pink staining" cells (ie. those closest to the lumen of the ductule) were examined, the distribution was similar in all groups and there was no obvious effect of parity or the cycle. Women taking oral contraceptives had a similar distribution to that in normally cycling women, and the small numbers meant that the slight trend towards larger cells among nulliparae was not significant.

TABLE 4:53 Size of epithelial cells of ductules:

Variation between specimens from the same patient

a) "Pink staining" cells

Patient Data	Right breast		Left breast	
	Specimen A	Specimen B	Specimen A	Specimen B
Nullip, follic			small	small
Nullip, on Pill	medium		large	
Nullip, luteal	small	small		
Nullip, luteal	small	small		
Nullip, luteal			medium	medium
Nullip, luteal	medium		medium	

b) "Clear" cells

Nullip, follic			large	large
Nullip, on Pill	medium		medium	
Nullip, luteal	medium	medium		
Nullip, luteal	large	large		
Nullip, luteal			large	large
Nullip, luteal	large		large	

TABLE 4:54 Size of epithelial cells of ductules:

Effects of parity and menstrual cycle, and oral contraceptives

a) "Pink staining" cells

		Size of cells		
		Large	Medium	Small
Nulliparae (34):				
Proliferative phase	(11)	1	6	4
Luteal phase	(16)	3	9	4
On oral contracept.	(7)	3	4	0
Parous (58):				
Proliferative phase	(27)	1	16	10
Luteal phase	(24)	5	12	7
On oral contracept.	(7)	1	4	2
Total examined	92	14	51	27

b) "Clear" cells

Nulliparae (34):							
Proliferative phase	(11)	4)		6)		1)	
Luteal phase	(16)	12)	18	4)	13	0)	3
On oral contracept.	(7)	2)		3)		2)	
Parous (58):							
Proliferative phase	(27)	4)		13)		10)	
Luteal phase	(24)	3)	10	8)	24	13)	24
On oral contracept.	(7)	3)		3)		1)	
Total examined	92	28		37		27	

The difference in distribution of "clear" cells between nulliparae and parous women is highly significant (X^2: 16.7; $P < 0.001$)

When "clear cells" were examined, it was found that these were larger among nulliparae than among parous women. This trend was highly significant ($P < 0.001$). However, the distribution in the two halves of the cycle was similar, in both nulliparae and parous women, and the distribution among Pill-users was similar to that among normally cycling women. The greater numbers of large clear cells among nulliparae are associated with the increased amounts of Type 1 epithelium among nulliparae, since Type 1 epithelium is characterised by a complete or almost complete basal layer of clear cells (as discussed in the previous section).

(iii) Other factors (Tables 4:55 and 4:56) -

Menstrual age: Patients were divided into groups as before and no differences were found in the distribution of "pink-staining" cells among the different groups (Table 4:55). When "clear" cells were examined, however, the youngest age group (menstrual age 1-9 years) had a preponderance of large clear cells. Again, this appears to be due to the greater numbers of nulliparae (with Type 1 epithelium) in this age group.

Birth interval: When parous women were divided into groups according to birth interval, no differences in the distribution of either type of cell were seen (Table 4:55).

Breast-feeding history: Parous women were divided into groups according to whether or not they had breast-fed, and for how long. The slight differences in the distribution of "pink-staining" and "clear" cells shown in Table 4:56 are not significant, although there is a suggestion that "large" cells of either type are seen less frequently among women who have breast-fed for longer periods.

TABLE 4:55 Size of epithelial cells of ductules:

Effects of menstrual age and birth interval

a) "Pink staining" cells

		Size of cells		
		Large	Medium	Small
Menstrual age:				
1 - 9 years	(19)	2	13	4
10 -19 years	(28)	4	17	7
20 -29 years	(24)	7	8	9
30+ years	(16)	1	11	4
TOTAL EXAMINED	87	14	49	24
Birth interval:				
2 - 5 years	(5)	0	3	2
6 -10 years	(23)	5	11	7
11 -15 years	(19)	2	10	7
16 -20 years	(6)	0	3	3
TOTAL EXAMINED	53	7	27	19

b) "Clear" cells

Menstrual age:				
1 - 9 years	(19)	11	8	0
10 -19 years	(28)	7	10	11
20 -29 years	(24)	7	9	8
30+ years	(16)	1	8	7
TOTAL EXAMINED	87	26	35	26
Birth interval:				
2 - 5 years	(5)	1	1	3
6 -10 years	(23)	4	10	9
11 -15 years	(19)	4	9	6
16 -20 years	(6)	0	2	4
TOTAL EXAMINED	53	9	22	22

TABLE 4:56 Size of epithelial cells of ductules:

Effect of history of breast feeding among parous women

a) "Pink Staining" cells

		Size of cells				
		Large	Medium		Small	
Never breast-fed	(21)	4	10		7	
Breast-fed for 1 - 6 weeks	(10)	3	5		2	
8 - 20 "	(7)		1		6	
				19		11
24 - 46 "	(11)		9		2	
52 - 84 "	(5)		4		1	
TOTAL EXAMINED: 54		7	29		18	

b) "Clear" cells

Never breast-fed	(21)	6	8		7	
Breast-fed for 1 - 6 weeks	(10)	1	4		5	
8 - 20 "	(7)	0	2		5	
		3		15		15
24 - 46 "	(11)	2	8		1	
52 - 84 "	(5)	0	1		4	
TOTAL EXAMINED: 54		9	23		22	

In each table the distribution of cell types is similar in each group of patients

e) Secretion in lumina of ductules

(i) Variation - Multiple specimens were obtained in eight cases. In two of these cases, two specimens were obtained from the same breast, and in five cases specimens were obtained from right and left breasts. In the remaining case three specimens were obtained from each breast at mammoplasty. The results are shown in Table 4:57. In five cases there is complete agreement, and in each of the three remaining cases (including the multiple specimen) the disagreement was by only one grade, (eg. +++ compared with ++, or + compared with $\pm$).

(ii) Effects of parity and the cycle, and of oral contraception (Tables 4:58 and 4:59) -

Patients were divided into groups according to parity, stage of the cycle and use of oral contraception. The results are shown in Table 4:58. Although a slightly greater proportion of parous women than nulliparae had large amounts of secretion present (++ and +++), this difference was not significant.

In both parous and nulliparous women there was no difference between the stages of the cycle, and oral contraceptive users had a similar distribution of secretion to normally cycling women. The amount of secretion in the coded specimens was also checked, in an effort to check for bias. These specimens were chosen to represent different groups, but the code was not broken until all the assessments had been made. The results are shown in Table 4:59. There is a trend towards larger amounts of secretion among parous women, but in neither parous women nor nulliparae is there any obvious difference between the stages of the cycle. The large amounts of secretion seen in nulliparae taking oral contraceptives were not confirmed in the larger series.

TABLE 4:57 Assessment of secretion in lumina of ductules

Variation between specimens from the same patient

	Right breast		Left breast	
PATIENT DATA:-	Specimen A	Specimen B	Specimen A	Specimen B
Parous, follic	+		+	
Nullip, on Pill	+		+	
Nullip, luteal	+	+		
Nullip, luteal			+++	++
Nullip, luteal	+		+	
Parous, on Pill	+		+	
Parous, follic	±		+	
Nullip, on Pill	±	+	+	±
	+		+	

TABLE 4:58 Assessment of secretion in lumina of ductules

Effects of parity, stage of cycle and oral contraception

Amount of secretion (and number of patients)		NULLIPARAE (n:45)			PAROUS WOMEN (n: 84)		
		On Pill	Prolif phase	Luteal phase	On Pill	Prolif phase	Luteal phase
0	(11)	2	1	3		3	2
±	(22)	3	3	4	2	6	4
+	(56)	8	6	6	7	19	10
++	(27)	1	1	3	1	12	9
+++	(13)	1	1	2	2	3	4
TOTAL	129	15	12	18	12	43	29

SIMPLIFIED TABLE:

	Nulliparae	Parous women
0 and ±	16 (36%)	17 (20%)
+	20 (44%)	36 (43%)
++ and +++	9 (20%)	31 (37%)
	45 (100%)	84 (100%)

The difference between nulliparae and parous women is not significant ($x^2 = 5.41$).

TABLE 4:59 Assessment of secretion in lumina of ductules

Coded specimens: Effects of parity, stage of cycle and oral contraception

Nulliparae

Follicular phase		Luteal phase		On Pill	
(a)	±	(f)	0	(k)	+++
(b)	+	(g)	±	(l)	+++
(c)	+	(h)	++		
(d)	±	(i)	0		
(e)	0	(j)	±		

Parous women

(m)	++	(p)	++	(v)	±
(n)	±	(q)	++	(w)	+
(o)	+	(r)	++		
		(s)	++		
		(t)	±		
		(u)	++		

(iii) Other factors (Table 4:60 and 4:61) - Menstrual age: Patients were grouped according to menstrual age with each grouping covering a ten-year age range. The results (Table 4:60) show no difference in distribution of secretion between any of the groups.

Birth interval: Patients were grouped according to the number of years between menarche and the first full-term pregnancy, with each group covering a five-year range. The results (Table 4:60) show no significant difference in distribution of secretion in any of the groups.

Breast-feeding history: Parous women were grouped according to whether or not they had ever breast-fed, and if so, for how long. The results (Table 4:61) show no difference between breast-feeders and those who had never breast-fed, and no effect of the total duration of breast-feeding.

TABLE 4:60 Assessment of secretion in lumina of ductules

a) Effect of menstrual age

Amount of secretion (and numbers of patients)		MENSTRUAL AGE (years)			
		1 - 9	10 - 19	20 - 29	30+
0	(11)	1	3	6	1
±	(22)	4	9	5	4
+	(58)	8	14	20	11
++	(27	2	9	11	5
+++	(13)	3	6	1	3
TOTAL	126	18	41	43	24

b) Effect of birth interval among parous women

Amount of secretion (and numbers of patients)		BIRTH INTERVAL (years)				
		1 - 9	6 - 10	11 - 15	16 - 20	21+
0	(5)		2	3		
±	(11)		3	7	1	
+	(33)	1	15	14	3	
++	(22)	2	10	7	3	
+++	(9)	1	1	5	1	1
TOTAL	80	4	31	36	8	1

In each table, distribution is similar in each group of patients

TABLE 4:61

Assessment of secretion in lumina of ductules:

Effect of breast-feeding history among parous women

Amount of secretion (and number of patients)		Never breast-fed	Length of breast-feeding				All breast-feeders
			1-6 wks	8-20 wks	24-46 wks	52-84 wks	
0	(5)		2	3			5
±	(12)	3	2		5	2	9
+	(31)	14	4	5	3	5	17
++	(19)	9	1	4	4	1	10
+++	(6)	2	1		3		4
TOTAL	73	28	10	12	15	8	45

Distribution is similar in each group of patients

D Examination of the stroma

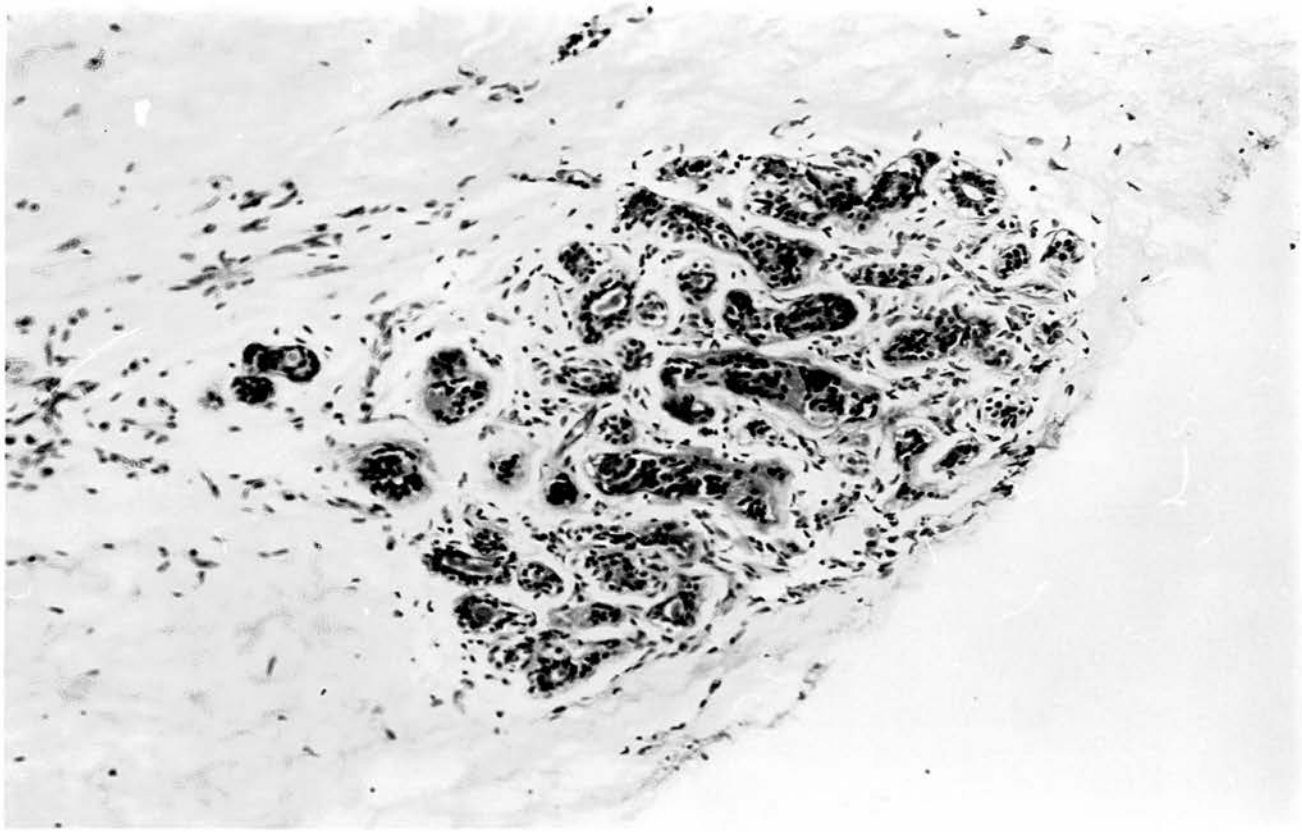
1 Methods

Cyclical changes have previously been described in both the intralobular and the extralobular stroma.

a) Intralobular stroma Three aspects of the intralobular stroma had received attention from previous investigators - the looseness of the stroma (Taylor 1936; Foote and Stewart 1945; Dabelow 1957; Nizze 1972), the amount of cellular infiltrate (Foote and Stewart 1945; Lewis and Geschickter 1934; Dabelow 1957), and the mucopolysaccharide content (Ozzello and Speer 1958). Each of these three was assessed separately in this investigation.

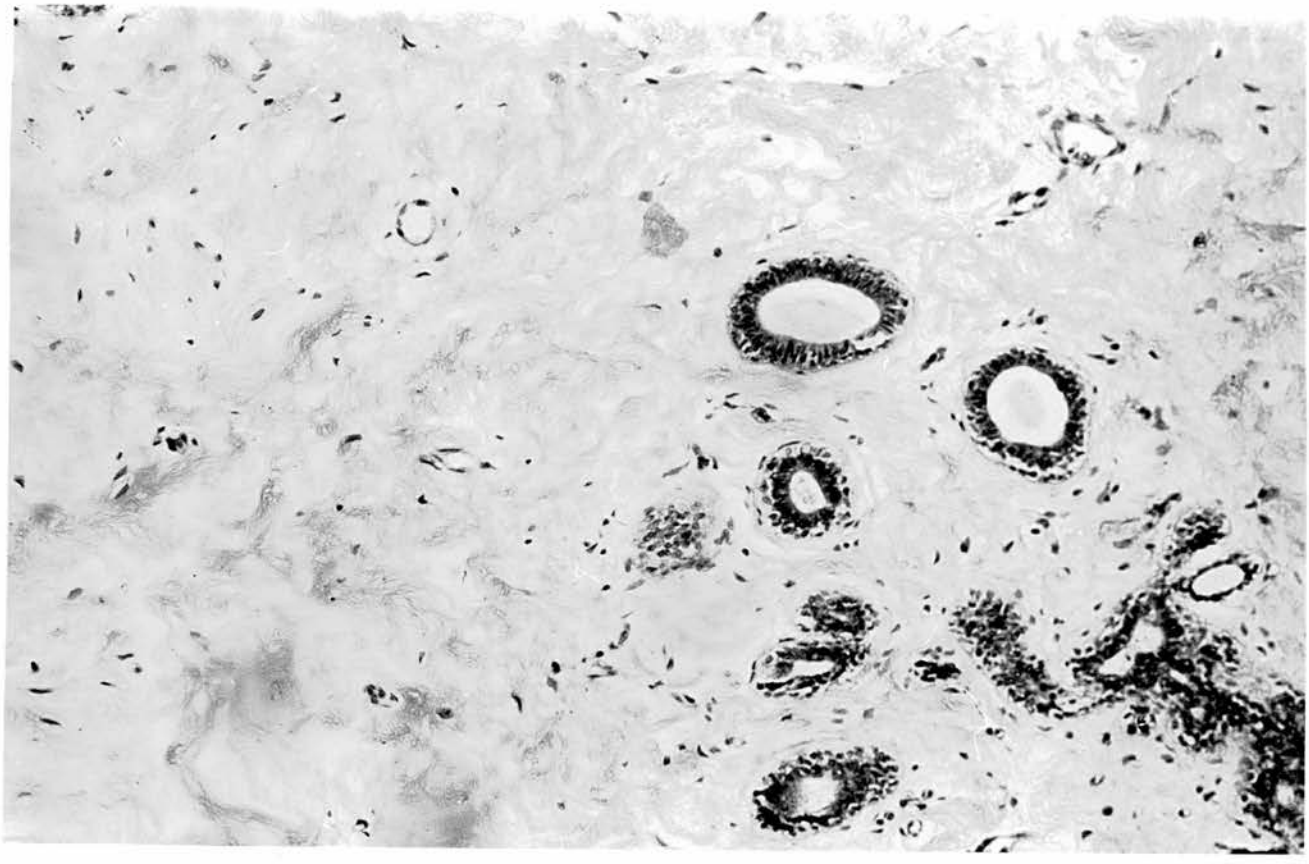
(i) Cellularity - Using sections stained with haematoxylin and eosin, sections were graded according to a subjective overall assessment of the amount of cellular infiltration of the intralobular stroma. Account was taken of variation between lobules, but a single overall assessment was made. Five gradings were used: low (Fig 4:13), low/moderate, moderate, moderate/high, and high (Fig 4:12).

(ii) Oedema - The intralobular stroma frequently stained more lightly than the extralobular stroma (see Fig 4:1b), and this appears to be due to a looseness of the fibrous tissue. This looseness was assessed using five grades. Grade "0" meant that the intralobular stroma was indistinguishable from the extralobular stroma (Fig 4:14). Grade "±" meant they were only just distinguishable. Grade "+" meant they were distinguishable in most lobules. Grade "++" meant that there was a fairly marked separation of fibres in all lobules. Grade "+++" meant there was wide separation of fibres in all lobules (Fig 4:1b).



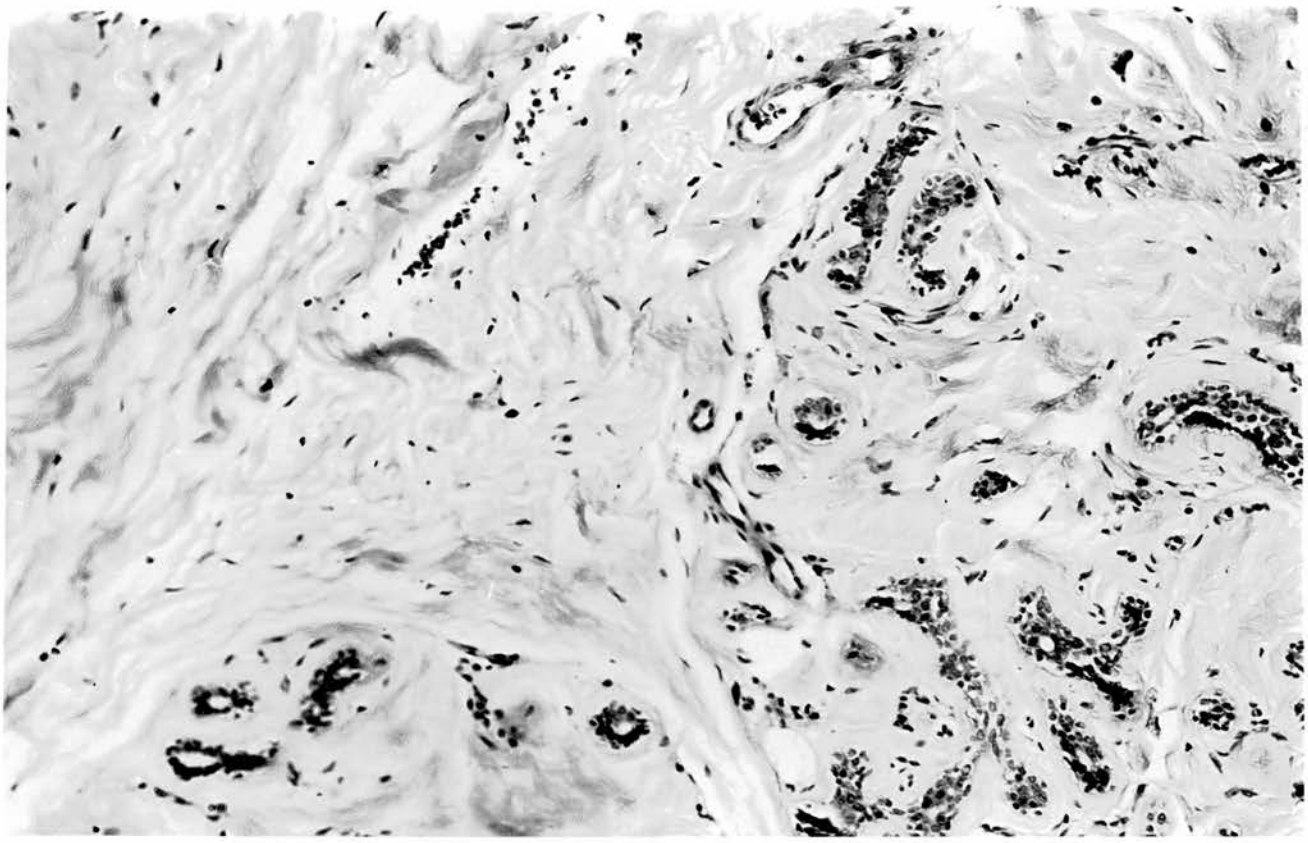
x 200

Fig 4:12 To illustrate cellular intralobular stroma (graded +++) The specimen was obtained at biopsy (diagnosis of lump: fibroadenoma) from a 52 year-old nulliparous woman 14 days after her period. The date of her subsequent period is not recorded. The plasma progesterone concentration was 1.71 ng/ml. Her menarche had been at age 13, and her periods had been regular (3/28) until three months before the biopsy was taken.
Compare Fig 4:13



x 200

Fig 4:13 To illustrate acellular intralobular stroma (graded ±)
The specimen was obtained at biopsy (diagnosis of lump: fibroadenosis) from a 41 year-old nulliparous woman on the 29th day of a 31-day cycle. The plasma progesterone concentration was not measured as the specimen tube broke. Her menarche had been at age 14. She had never been on oral contraception.
Compare Fig 4:12



x 200

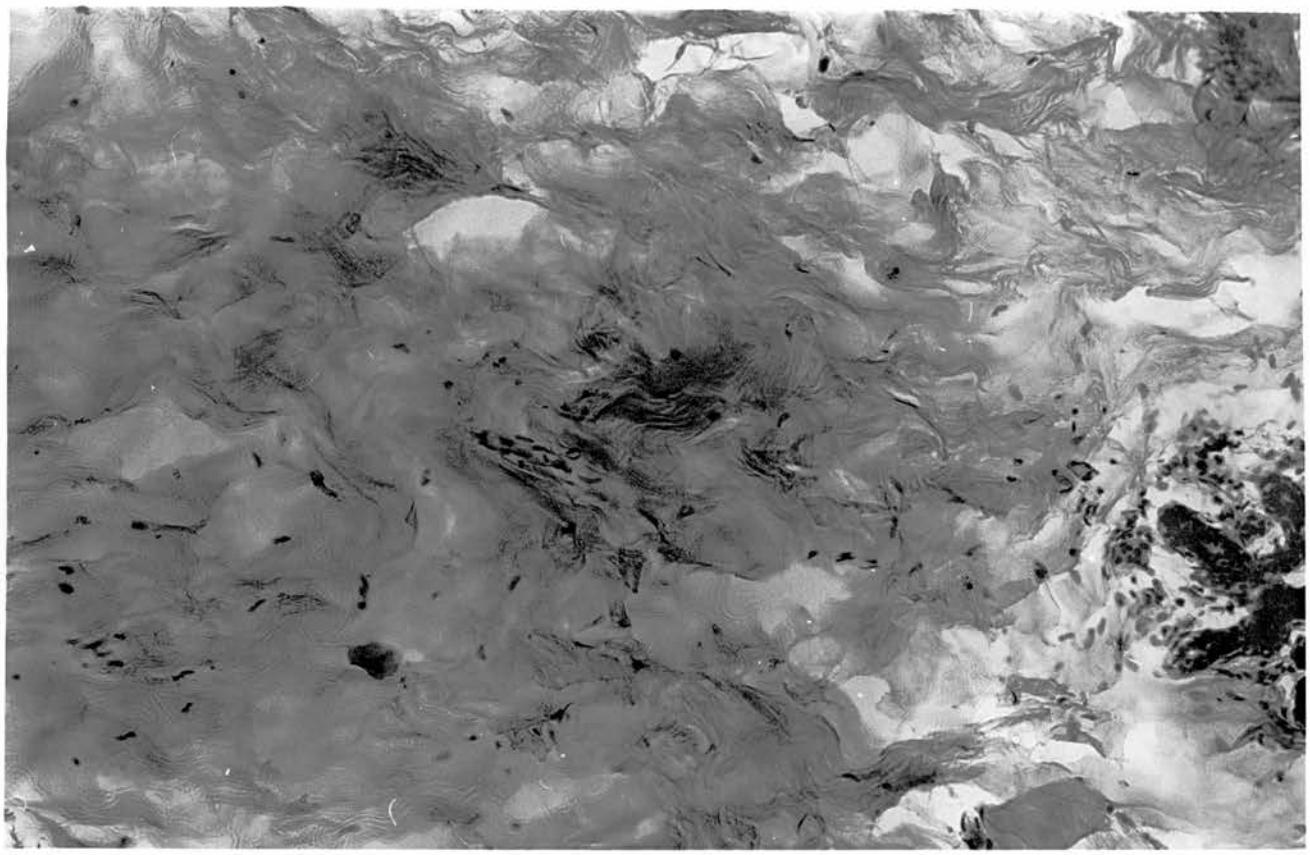
Fig 4:14 To illustrate lobules without stromal oedema
The specimen was obtained at biopsy (diagnosis of lump: fibroadenosis with cysts and epitheliosis) from a 32 year-old woman on the tenth day of her cycle. The date of her subsequent period is not recorded. The plasma progesterone concentration was "undetectable". Her menarche had been at age 11. She had never taken oral contraceptives.
Compare Fig 4:1b

(iii) Acid mucopolysaccharide content - When sections were stained with alcian blue and periodic acid Schiff there was marked variation in the amount of blue staining of the intralobular stroma - indicating variation in the amount of acid mucopolysaccharide present. (There was very little variation in the amount of neutral polysaccharide, indicated by pink staining with this stain.) The "blueness" of the stroma was assessed subjectively as follows:

0	no staining
±	Staining only just detectable
+	staining clearly seen in some lobules
++	most lobules showed obvious staining
+++	all lobules showed obvious staining

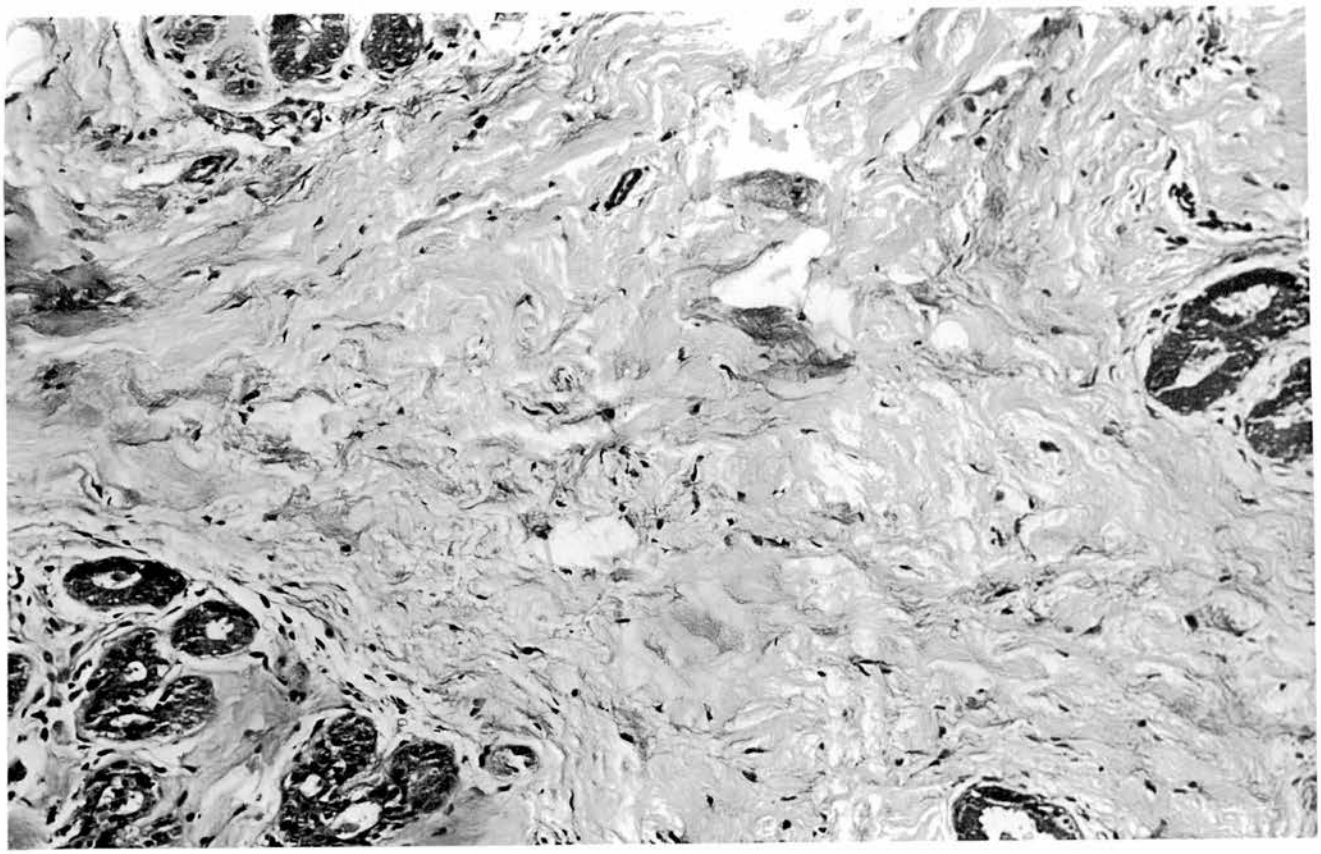
b) Extralobular stroma The appearance of the extralobular stroma is said to show cyclical changes (Huseby and Thomas 1954). In the present investigation, its appearance on haematoxylin and eosin staining was assessed according to whether it stained deeply, moderately or lightly, and whether the staining was uniform. Non-uniform staining appeared to be due to separation of the fibres of the stroma, possibly an indicator of stromal oedema. The appearance was graded as "thin", "normal", or "thick" (Fig 4:15) by the intensity of the staining, or as "fibrous" if the stain was obviously not uniform but was stringy in appearance (Fig 4:16). There was not much variation of the appearance over each section, and a single assessment was made.

All the stromal assessments were made without the observer knowing the parity or stage of cycle of the subjects. The specimens were not, however, re-numbered for these assessments because the observer's repeated examinations would have allowed him to recognise sections even if they had been re-coded between each part of the examination. The large number of sections involved meant that it was impossible for the observer to remember details of parity of more than one or two subjects.



x 200

Fig 4:15 To illustrate extralobular stroma graded as "thick"
The specimen was obtained at mammoplasty from a 45 year-old woman on the 24th day of a 25-day cycle. The plasma progesterone concentration was 4.307 ng/ml. She had experienced the menarche at age 13, and had had one full-term pregnancy when she was 22 years old. She had breast-fed for six weeks. She had taken an oral contraceptive for about four years until four months before the operation. She complained of breast heaviness for a week before her periods, but this had now become continuous and the indication for the operation was heaviness and lumpiness of the breasts.



x 200

Fig 4:16 To illustrate extralobular stroma graded as "fibrous"
The specimen was obtained at biopsy (diagnosis of the lump: fibroadenoma) from a 33 year-old woman on the 22nd day of her cycle. The date of her subsequent period is not recorded: her cycle varied in length from 2-3/21 to 2-3/35 days. The plasma progesterone concentration was 6.79 ng/ml. Her menarche had been at age 12. She had had two full-term pregnancies, the first when she was 22 years old and the second at age 23. She had not breast-fed.

2 RESULTS

a) Variation (Table 4:62)

Cellularity: Four pairs of specimens, each pair from the same breast, and three pairs from right and left breasts were studied. Exact agreement between assessments was found in each pair.

Oedema: Five single-breast pairs of specimens and four pairs from right and left breasts were studied. Agreement was exact in all but three pairs: in two right/left pairs there was disagreement by one grade, and in one single-breast pair, disagreement by two grades (O compared with +).

Acid mucopolysaccharide content: Three single-breast and five right/left pairs were studied, along with multiple samples from each breast of two patients undergoing mammoplasty. Agreement was exact in all but one pair: in the remaining pair disagreement was by one grade. In the multiple specimens, disagreement was by only one grade.

Extralobular stroma: In four single-breast pairs and three right/left pairs, agreement was exact in five, and differed by one grade in the remaining two pairs (both of them single-breast pairs).

b) Effects of parity and the cycle

(i) Cellularity (Table 4:63) - Over all patients, the difference between the follicular and luteal phases was minimal, but when the week of the cycle was taken into account (Table b), the difference between week 2 and week 4 was significant ($X^2 = 6.42$; $P<0.05$).

TABLE 4:62 Histological appearance of the stroma:

Variation between specimens from the same patient

a) Cellularity

	Pt 8	Pt 51	Pt 94
Right breast	Mod	Mod	Mod/low
Left breast	Mod	Mod	Mod/low

	Pt 35	Pt 53	Pt 74	Pt 92
1st specimen	Low	Mod	Mod/high	Mod
2nd specimen	Low	Mod	Mod/high	Mod

b) Oedema

	Pt 8	Pt 51	Pt 94	Pt 132
Right breast	0	0	+	±
Left breast	+	0	+	±

	Pt 23	Pt 35	Pt 36	Pt 53	Pt 74	Pt 92
1st specimen	+	0	++	0	0	++
2nd specimen	+	0	+++	0	0	+

c) Acid mucopolysaccharide content

	Pt 8	Pt 51	Pt 94	Pt 132	Pt 168
Right breast	±	0	+	0	0
Left breast	±	0	+	0	±

	Pt 53	Pt 74	Pt 92
1st specimen	0	0	+
2nd specimen	0	0	+

	Pt 150a	Pt 150b	Pt 150c	Pt 163a	Pt 163b	Pt 163c
Right breast	±	0		±	±	0
Left breast	0	±	±	±	±	±

d) Extralobular stroma

	Pt 8	Pt 51	Pt 94
Right breast	Fibrous	Fibrous	Normal
Left breast	Fibrous	Fibrous	Normal

	Pt 35	Pt 53	Pt 74	Pt 92
1st specimen	Thick	Normal	Fibrous	Normal
2nd specimen	Fibrous	Normal	Fibrous	Fibrous

TABLE 4:63

Cellular infiltrate in intralobular stroma: effects of parity and cycle

a) All patients (n:113)

			Cell counts				
			High	Mod/high	Mod	Mod/low	Low
Nulliparae:	prolif	(12)	1	-	4	2	5
	luteal	(17)	1	3	10	2	1
Parous:	prolif	(37)	5	7	16	6	3
	luteal	(30)	2	6	14	6	2
On Pill:	nullip	(7)	1	1	3	1	1
	parous	(10)	4	2	4		

b) All patients except those on oral contraceptives (n:94)

Week of cycle					
Week 1	1	3	10	3	2
Week 2	5	0	12	5	7
Week 3	3	1	8	4	0
Week 4	0	13	12	3	2

c) Nulliparae

	High & Mod/high	Mod	Low & Mod/low
Week 1	0	4	1
Week 2	1	2	5
Week 3	1	0	0
Week 4	3	7	3

d) Parous

Week 1	4	6	4
Week 2	4	10	7
Week 3	3	8	4
Week 4	10	5	2

In Tables (b) and (d) the difference in distribution of cell counts between week 2 and week 4 are significant ($P<0.05$).

(X^2 = 6.42 and 6.67 respectively)

There is no obvious difference between parous and nulliparous women in the amount of cellular infiltrate. Among nulliparae, high counts were more frequent in the luteal phase of the cycle, and low counts in the proliferative phase, but the difference was small - perhaps because of the small numbers of specimens available for examination. When the week of the cycle was taken into account, the cyclical change was not any more pronounced, and was not statistically significant.

Among parous women, there was no difference between proliferative and luteal phases of the cycle, but when specimens were examined according to the week of the cycle, there was a slight increase in the number of high counts in week 4. The difference between week 2 and week 4 was statistically significant ($X^2 = 6.67$: $P<0.05$).

When women taking oral contraceptives were examined, the distribution of counts among nulliparous women was similar to that among normally cycling women, but among parous women there was a tendency to higher cell counts.

(ii) Oedema (Tables 4:64 and 4:65) - No difference emerged between parous and nulliparous women, or between stages of the cycle in either group. The fact that "+++" gradings were seen only among parous women probably reflects the greater numbers of parous women examined. Women taking oral contraceptives had a similar distribution of gradings as normally cycling women.

(iii) Acid mucopolysaccharide content (Table 4:66) - Again all groups showed the same distribution of gradings, and no differences attributable to stage of the cycle, parity, or oral contraception could be detected.

TABLE 4:64

Oedema of intralobular stroma: Effects of parity, stage of cycle and oral contraception

			+++	++	+	±	0
Nulliparae:	Proliferative phase	(18)		1	1	8	8
	Luteal phase	(15)		1	5	2	7
Parous:	Proliferative phase	(45)	1	7	13	17	7
	Luteal phase	(31)	2	5	11	9	4
Taking "Pill":	Nulliparae	(9)		2	3	1	3
	Parous	(13)			4	8	1

Total: 131 specimens from 121 patients

+++ : Wide separation of fibres in intralobular stroma in all lobules

++ : Fairly marked separation of fibres

+ : Intralobular stroma distinguishable from extralobular stroma in most lobules

± : Intralobular stroma only just distinguishable from extralobular stroma

0 : Intralobular stroma indistinguishable from extralobular

TABLE 4:65 Oedema of Intralobular stroma

Coded specimens: Effects of parity,

stage of cycle and oral contraception

Nulliparae

Follicular phase		Luteal phase		On Pill	
(a)	+++	(f)	+++	(k)	+
(b)	-	(g)	-	(l)	+++
(c)	+	(h)	-		
(d)	+++	(i)	+++		
(e)	+	(j)	+++		

Parous

(m)	+++	(p)	+	(v)	-
(n)	+	(q)	+++	(w)	+
(o)	-	(r)	+		
		(s)	-		
		(t)	-		
		(u)	+		

"+++" means intralobular stroma clearly seen

"+" means intralobular stroma just seen

"-" means not seen

TABLE 4:66

Acid mucopolysaccharide content of intralobular stroma: Effects of parity, stage of cycle and oral contraception

			+++	++	+	±	0
Nulliparae:	Proliferative phase	(25)		1	2	9	13
	Luteal phase	(19)		1	5	4	9
Parous:	Proliferative phase	(54)	1	7	7	13	26
	Luteal phase	(34)		6	7	8	13
On Pill:	Nulliparae	(16)	1	1	1	6	7
	Parous	(16		1	1	3	11

TOTAL: 164 specimens from 146 patients

Acid mucopolysaccharide content assessed by subjective grading of blue staining of intralobular stroma by Alcian Blue/Periodic Acid Schiff stain.

+++ all lobules showed obvious staining

++ most lobules showed obvious staining

+ staining clearly seen in some lobules

± staining only just detectable

0 no staining

TABLE 4:67

Extralobular stroma: Appearance of H & E staining: Effects of parity, stage of cycle and oral contraception

			Thick	Fibrous	Normal	Thin
Nulliparae:	Follicular phase	(14)	4	3	6	1
	Luteal phase	(17)	0	4	11	2
Parous:	Follicular phase	(38)	12	8	13	5
	Luteal phase	(29)	6	12	9	2
All Follicular phase		(52)	16	11	19	6
All Luteal phase		(46)	6	16	20	4
On Pill:	Nulliparae	(10)	2	2	5	1
	Parous	(8)	1	1	6	0

"Thin": poor staining, but uniform

"Thick": heavy staining, again uniform

"Fibrous": stain broken into strands, with clear areas between

The difference in distribution of "thick" and "fibrous" stroma between the Follicular and Luteal phases of the cycle approaches statistical significance ($X^2 = 3.80$) but does not reach significance. ($P>0.05$)

(iv) Extralobular stroma (Table 4:67) - Parous and nulliparous women showed a similar distribution of gradings, but there was a suggestion of a change during the cycle, with "thick" stroma being seen more frequently in the proliferative phase in both groups, and "fibrous" (ie. more oedematous) stroma being seen more frequently in the luteal phase. This difference is only marginal, when the numbers of specimens graded as "normal" and "thin" are borne in mind, but it may be a reflection of the oedema of the breast which can be observed macroscopically (see Chapter 3) in the luteal phase.

c) Other factors

Effects of menstrual age and birth interval (Tables 4:68 and 4:69) - The menstrual age of each patient was calculated as her age minus her age at menarche. Birth interval was the difference between her age when she had her first full-term pregnancy, and her age at menarche. Each of these was compared with the distribution of gradings of cellularity, oedema and acid mucopolysaccharide content, and no difference could be detected.

When the extralobular stroma was examined, there was a suggestion that "thick" stroma was more frequently seen among older women (ie. women of greater menstrual age), but this difference was minimal.

Effects of breast feeding history (Table 4:70) - Among parous women, the total length of breast feeding as recalled by the patient was compared with the histological findings. Women who had never breast-fed showed a similar distribution of all the gradings as those who had breast-fed, and the length of time that breast-feeding had continued did not affect the distribution either.

TABLE 4:68 Effect of menstrual age on the stroma (ie. age minus age at menarche)

I INTRA-LOBULAR STROMA		1-9 yrs	10-19 yrs	20-29 yrs	30+ yrs
a) Cellularity:	low	1	2	6	1
	mod/low	4	4	6	3
	moderate	8	15	16	10
	mod/high	4	5	7	3
	high	2	6	2	4
Total examined:	109	19	32	37	21
b) Oedema:	0	6	8	10	3
	±	6	14	14	11
	+	6	12	8	10
	++	3	4	7	0
	+++	0	0	1	1
Total examined:	124	21	38	40	25
c) Acid mucopolysaccharide content:					
	0	12	20	25	20
	±	7	16	10	9
	+	7	10	4	2
	++	2	2	7	5
	+++	0	0	1	0
Total examined:	159	28	48	47	36
II EXTRA-LOBULAR STROMA					
	Thin	1	4	3	2
	Normal	12	14	18	6
	Fibrous	6	10	7	7
	Thick	2	5	9	6
Total examined:	112	21	33	37	21

TABLE 4:69 Effect of birth interval on breast stroma
(age at first birth minus age at menarche)

I INTRA-LOBULAR STROMA		2-5 yrs	6-10 yrs	11-15 yrs	16-20 yrs	20+ yrs
a) Cellularity:	low	2	1	2	0	0
	mod/low	0	3	7	1	0
	moderate	1	11	14	3	0
	mod/high	1	8	4	2	0
	high	1	4	3	3	0
Total examined:	71	5	27	30	9	0
b) Oedema:	0	0	2	9	0	0
	±	2	16	11	5	0
	+	3	13	9	2	0
	++	1	3	3	1	0
	+++	0	0	1	1	0
Total examined:	82	6	34	33	9	0
c) Acid mucopolysaccharide content:						
	0	4	21	14	7	2
	±	1	8	13	1	0
	+	3	5	6	1	0
	++	1	5	5	1	0
Total examined:	98	9	39	38	10	2
II EXTRA-LOBULAR STROMA						
	Thin	1	1	3	1	
	Normal	1	13	7	4	
	Fibrous	2	8	11	2	
	Thick	1	5	9	2	
Total examined:	71	5	27	30	9	

TABLE 4:70 Effect of breast-feeding history on the stroma among parous women

I	INTRA-LOBULAR STROMA		Never breast-fed	1-6 wks	8-20 wks	24-46 wks	52-84 wks
a)	Cellularity:	low	1	1	2	1	0
		mod/low	5	3	0	1	0
		moderate	13	3	2	4	6
		mod/high	6	4	3	2	0
		high	2	1	0	5	2
	Total examined:	67	27	12	7	13	8
b)	Oedema	0	4	3	1	3	0
		±	13	3	8	4	3
		+	7	6	2	5	4
		++	4	0	1	2	1
		+++	1	1	0	0	0
	Total examined:	76	29	13	12	14	8
c)	Acid mucopolysaccharide content						
		0	20	7	10	7	4
		±	6	5	1	5	2
		+	7	2	1	4	1
		++	3	3	2	2	1
		+++	1	0	0	0	0
	Total examined:	94	37	17	14	18	8
II	EXTRA-LOBULAR STROMA						
		Thin	3	3	0	0	0
		Normal	9	5	2	3	4
		Fibrous	9	1	2	5	3
		Thick	6	3	3	5	1
	Total examined:	67	27	12	7	13	8

d) Relationship between stromal changes and breast symptoms

The stromal appearances were compared in patients who had complained of breast symptoms during or before menstruation, and those who had no symptoms. The results are presented in Table 4:71. The amount of stromal oedema did not differ significantly between the two groups, although there was a slight trend towards more pronounced oedema of the intralobular stroma among women with symptoms. The difference between the two groups of women was more pronounced when dilatation of the ductule lumina was assessed, but the difference was not significant. Again, however, there was a trend towards greater dilatation among women with symptoms.

The distribution of gradings of the appearance of the extralobular stroma was similar in both groups, with a possible trend towards more "thick" and "fibrous" stroma among women with symptoms.

IV AUTOPSY CASES

Attempts were made to obtain breast tissue from victims of sudden death, in the hope that this would avoid the problem inherent in the use of biopsy material - that the patient has some complaint related to her breasts necessitating operation. Eight specimens were obtained, with the very kind co-operation of the forensic pathologists acknowledged at the introduction to this thesis. Details of the eight cases are shown in Table 4:72. It is apparent from this table that essential details (such as parity and oral contraceptive use) were unobtainable. Because of the social circumstances of these patients, many of whom had comitted suicide, it was impossible to trace details of contraceptive use from Family Doctor records. Although the ovaries were examined, accurate staging of the cycle proved impossible (the endometrium had frequently undergone autolysis). For all these reasons, this source of material had to be abandoned.

TABLE 4: 71 Histological appearances in patients with premenstrual breast symptoms

A	Oedema of intralobular stroma		with symptoms	no symptoms
		+++	1	
		++	1	4
		+	18	6
		±	23	14
		0	5	6
	TOTAL	78	48	30
B	Assessment of ductule lumina			
		Dilated	5	1
		Normal/dilated		2
		Normal	22	8
		Small/normal	2	
		Small	14	12
	TOTAL	66	43	23
C	Appearance of extralobular stroma			
		Thick	8	1
		Fibrous	14	6
		Thin	3	1
		Normal	21	14
	TOTAL	68	46	22

Differences in distribution are not significant

TABLE 4:72 Autopsy Cases

Case	Age	Parity	Cause of death	Contraception	Other points
1	25	0?	?drug o/d	IUCD	
2	35	0?	Alcohol o/d	?	Mastitis
3	20	1+0	Trauma	?	CL present
4	30	2+0	SBE	sterilised	CL present
5	29	0	Drowning	?	Nullip cervix
6	45	?	Barbiturate o/d	? none	
7	24	1+0	Inhaled vomit after a fit	IUCD	
8	39	3+?	Coronary thrombosis	On pill for several years	First pregnancy at age 17

o/d Overdose

SBE Subacute bacterial endocarditis

IUCD Intrauterine contraceptive device

CL Corpus luteum

V DISCUSSION

A Summary of positive findings

1 Differences between parous and nulliparous women

a) Lobules per unit area Parous women had a greater density of lobules than did nulliparae ($P<0.05$). (Table 4:22).

b) Ductule density within lobules The number of ductules per unit area within lobules was greater among parous women than among nulliparae ($P<0.05$). (Table 4:22).

c) Epithelial types "Type 1" epithelium was commoner among nulliparae than among parous women, but "Type 3" epithelium was commoner among parous women than nulliparae ($P<0.001$). (Table 4:44)

d) Size of "clear" epithelial cells Clear basal epithelial cells were graded as "large" more frequently among nulliparae than among parous women ($P<0.001$). This highly significant difference reflects the greater frequency of "Type 1" epithelium among nulliparae.

2 Differences due to the menstrual cycle

a) Epithelial height During the menstrual cycle there was a variation in the height of the ductule epithelium as assessed by direct measurement. This was greater in the luteal phase of the cycle than in the follicular phase. The significance of this difference was greater among parous women ($P<0.01$) than among nulliparae ($P<0.05$).

b) Cellular infiltrate of stroma When the week of the cycle was taken into account, a cyclical variation in cellular infiltration of the intralobular stroma was seen. There was a greater number of cells during week 4 of the cycle than during week 2 ($P<0.05$).

3 Differences due to menstrual age

a) Lobules per unit area and ductules per unit area The density of both lobules and ductules was less among women within ten years of the menarche than among older women ($P<0.05$). (Table 4:26)

b) Diameter of ductule lumina The lumina of the ductules tended to become smaller with increasing menstrual age ($P<0.05$). (Table 4:39).

B Discussion

Sir Astley Cooper said of the milk cells, "Their number is very great; it varies much and it would be an act of folly and inutility to endeavour to reckon them". (Cooper 1840). This pessimistic view has to some extent been justified by the fact that for the last fifty years controversy has surrounded the question of the breast's response to the normal menstrual cycle. The variation between specimens, described in this chapter, partly explains this controversy. The controversy is unlikely to be explained by the difference between nulliparous and parous women, since these differences are not marked, but nevertheless no previous investigators have looked for such changes. The menstrual cycle appears to have little influence on breast histology, and other variables even less influence.

1 Variation between specimens

The lobular architecture is the area in which the greatest variation between specimens occurs. Some variables (such as the percentage area of lobules) trebled or quadrupled in one specimen compared to another from the same block (Table 4:19), and there was a slightly greater variation between different blocks taken from the same breast. Variation between right and left breasts was no more marked than variation between different blocks from a single breast. This amount of variability emphasises the pitfalls of drawing conclusions from a small number of specimens (as were originally drawn by Rosenburg, 1922). Large numbers of specimens have to be examined before trends can be discerned, and when this has been done by previous investigators, the conclusions have tended to be guarded (Foote and Stewart, 1945) or negative (Haagensen, 1971).

Although there is marked variation in lobular architecture, it appears from this study that less variation between specimens occurs in the structure of the epithelium of the ductules, and in the stroma (eg. Tables 4:31, 4:43, 4:62).

2 Variation associated with parity

The fact that variations due to parity had not previously been described (or even, apparently, looked for) was one of the main reasons for undertaking this study. It has been assumed by others that since the breast returns after involution to a structure resembling that of the virgin breast (Dawson 1935), the two must be identical. The results of the present study suggest, on the contrary, that parity has more important effects on breast histology than does any other factor - although even the effects of parity are far from obvious.

The finding that the density of lobules is greater among parous women than among nulliparae (Table 4:22) suggests that involution after pregnancy is not always complete. (None of the patients studied had recently breast-fed, and so the results are unlikely to be influenced by breasts in the process of involuting). However, the associated variable (the number of ductules per unit area) did not differ significantly. Nevertheless, the number of ductules per unit area tended to be greater among parous women, and although this trend did not reach significance it tends to support the conclusion that lobule numbers are greater among parous women.

The density of ductules within lobules also appeared greater among parous women, suggesting a similar process of incomplete involution. However, in this case the difference in the associated variables (average number of ductules per lobule, and area of average lobule) was only very slight, and this casts doubt on the significance of the

variation in ductule density. The size of the lobules is not changed after pregnancy and involution, and if ductules are present in greater numbers, the difference must be only very slight.

On the other hand, the difference in distribution of epithelial types is highly significant. Even here, however, the change brought about by parity is a subtle rather than a sharp one, and the increased frequency of Type 1 epithelium among nulliparae is seen only when a large number of specimens is examined (Tables 4:44 and 4:45). This would explain why this trend has not previously been reported, despite close attention being paid to the basal clear cells which characterise Type 1 epithelium (eg Bassler 1970). The reason for the difference in epithelial types is unknown, because the function of the basal clear cells is unknown. As discussed above (Chapter 2), it has been assumed by some workers that these cells are myoepithelial cells, but their large numbers in some specimens (Fig 4:5) makes this very unlikely. Since they are basal in position, they may be precursors of the pink-staining cells: if this is the case then the implication that they are less differentiated and are capable of proliferation has important implications when the breast's susceptibility to carcinogenesis is considered (Chapter 7).

3 Variation associated with the menstrual cycle

In this study the phase of the menstrual cycle was normally decided by the plasma concentration of progesterone. The advantage of this method is that it allows ovulatory cycles to be distinguished from anovulatory cycles, a distinction that has not been made by previous investigators. It also allows comparison to be made between cycles of unequal length, since variation in the day of ovulation may have an important influence on the effect that the cycle has on the breast. A possible disadvantage of this method of dividing the cycle into two

rather than four or more, is that a change in one of four phases might be obscured. If a change were missed it would be likely to be a small one, or to be compensated for by an opposite change in the same phase of the cycle. Such a phenomenon seems unlikely, and no previous investigation has suggested it: changes, if they occur, are said to be slow in onset and regression, and therefore are likely to be detected by the method chosen.

The lack of variation attributable to the menstrual cycle was a striking negative finding in this study. As described in Chapter 2, investigators studying small series of specimens had described marked changes, (eg. Rosenburg 1922). (For example, in the present study the difference in the appearance of Figs 4:3 and 4:4 could be attributed to the difference in plasma progesterone concentrations, since all other variables are almost identical). The results of the present study, however, agree with those investigators who looked critically at large series - for example, Haagensen (1971), who studied 400 specimens and concluded that cyclical changes do not occur in the lobules. Other investigators have suggested that changes occur although not all lobules respond to them (Foote and Stewart 1945): because of the variability of histology from different parts of the same breast this theory would be easy to illustrate, but there has been no supporting data in the form of quantitation of how many lobules show changes and how many do not. The theory that cyclical changes occur in some but not all lobules makes assumptions which are practically impossible to prove or disprove, but the burden of proof is, I suggest, on those who believe such a phenomenon occurs.

The one cyclical change in the ductules that was found in the present study was a change in epithelial height (Table 4:38). This is greater during the luteal phase of the cycle, and the difference is seen among both parous women and nulliparae. The greater significance of the variation among parous women ($P<0.01$) than nulliparae ($P<0.05$) is probably due to the difference in the number of specimens examined. The fact that an effect is seen among both parous and nulliparous women does not agree with the findings in Chapters 5 and 6, in which cyclical effects were seen among parous women but not among nulliparae. However, those chapters are concerned with functional aspects, and the relationship between epithelial height and function is not certain. The phenomenon observed here is similar to that reported in the ultrastructural study of Fanger and Ree (1974), who reported that epithelial cells became more active in the luteal phase of the cycle. The cellular infiltration of the intralobular stroma appeared to increase during week 4 of the cycle and was at its lowest during week 2. This finding agrees with the work of Foote and Stewart (1945), and also agrees to some extent with the finding in Chapter 6 of this thesis that numbers of plasma cells tend to increase during the latter part of the cycle (although this increase is inferred rather than directly observed in Chapter 6). The function of the cellular infiltrate remains uncertain: the suggestion that the cells are present to phagocytose desquamated epithelial cells seems unlikely in view of the finding that budding of the ductules and subsequent involution do not occur. As discussed in Chapter 6, the cellular infiltrate may reflect variation in secretion of a local transmitter produced by the epithelial cells, though this possibility remains speculative.

The results therefore suggest that progesterone does have an influence on the ductule epithelium, but that this influence is not sufficient to cause budding of the ductules. The observation that a biopsy taken during the seventh week of pregnancy (Fig 4:4) showed no increase in numbers of ductules or lobules - but did show increased epithelial height - supports the conclusion that cyclical changes during the normal menstrual cycle affect only the epithelium and not the lobules.

4 Influence of other factors

Dieckmann (1925) first suggested that the age of the subject was the main factor influencing the development of the lobules. This suggestion receives some support from the present study. In the present study, it was hoped that any changes would be more readily detected by subtracting the age at menarche from the subject's age, giving her "menstrual age", the reason for this being that lobular development does not begin until puberty. The results show that there is significantly less lobular density among specimens from women within ten years of the menarche than among specimens from older women. It seems likely that this is because lobular development is still taking place during the years just after the menarche - some of the specimens in the present study were obtained within a fairly short time (eg. four years) after the menarche. However, once a certain lobular development has been attained, no further development occurs, and there is no evidence in the present study to support Dieckmann's (1925) hypothesis that lobular development continues to occur during a woman's reproductive life.

On the contrary, the measurement of ductule diameters suggests the opposite - that a steady decrease in activity occurs during reproductive life. The progressive decrease in the mean luminal diameter found in this study, however, is not reflected by a decrease in any other variable,

and so its significance remains uncertain. It is possible that the degree of distension of ductule lumina normally depends on a low level of secretory activity by the epithelial cells, but if this is the case then the steady decrease of luminal diameter with age might be expected to be reflected by a decrease in the amount of stainable secretion or a decrease in epithelial height. Neither was found to be the case in this study.

Birth interval (ie. the difference between a woman's age at her first full-term pregnancy and her age at menarche) was studied because of the importance of this factor in the epidemiology of breast cancer (MacMahon 1972). However, it did not appear to have any influence at all on breast histology. This is not particularly surprising, and a possible explanation of the importance of the birth interval in breast cancer epidemiology is put forward in Chapter 7.

Breast-feeding history does not appear to influence histology. There is no evidence in the present study that prolonged breast-feeding is associated with, for example, incomplete involution. This is in agreement with clinical findings and with observations on the epidemiology of breast cancer, which also appear to show no influence of breast-feeding history (Chapter 7).

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Thus the histology of the breast shows marked variability, but is significantly influenced by parity. The menstrual cycle has an effect on the epithelium only, and not on the lobules. Menstrual age had only a slight effect. The next two chapters describe studies on the function of the epithelium, and the relevance of the changes attributable to parity is discussed in detail in the final chapter.

SUMMARY

Specimens of apparently normal breast tissue were obtained from 174 patients undergoing breast biopsy or mammoplasty. The histology of the lobules, the ductules and the stroma was examined. There was marked variation between specimens as far as lobular histology was concerned. Parous women showed slightly increased numbers of lobules per sq mm compared with nulliparae, but ductular epithelium showed a more significant variation between these two groups, with large clear basal cells being commoner among nulliparae. Gross cyclical changes in lobular histology with the menstrual cycle were not found: the influence of the menstrual cycle was limited to a change in the height of the ductule epithelium and a change in stromal cellularity. Increased epithelial height and increased cellularity of the intralobular stroma were found in the luteal phase among both parous women and nulliparae. Lobular development appeared incomplete soon after the menarche. Ductule diameters decreased with increasing age, but the patient's age did not otherwise influence breast histology. Breast feeding history and age at first pregnancy did not influence breast histology. Cyclical changes in the appearance of the stroma were minimal.

Chapter 5

DNA SYNTHESIS IN ORGAN CULTURE

A INTRODUCTION

The histological studies described in Chapter 4 suggested that the effects of parity and the menstrual cycle are seen in the epithelial cells of the ductules, rather than in gross lobular histological changes. Since histological changes during the cycle involved only the height of the epithelium, other methods of investigating epithelial cell activity were explored.

Two methods were used:- one was the assessment of immunoglobulin synthesis, which is described in Chapter 6, and which gives at best an indirect assessment of epithelial cell activity. The other method, described in this chapter, was direct study of epithelial cell activity in tissue-culture.

Cell turnover occurs to some extent in most tissue of the body. Its rate can be assessed in vitro by the incorporation of labelled precursors into the DNA of the cell nuclei as measured by autoradiography. An in vitro method of examination of mammary tissue was already in use in the Department of Clinical Surgery of Edinburgh University, for the study of neoplastic tissue. It had been set up by Dr J R W Masters, who agreed to collaborate on the study of normal tissue by the same method. In the results to be described, the preparation of autoradiographs and the counting of labelling indices were carried out by Dr Masters and his assistant, Miss Katharine Sangster.

B PATIENTS STUDIED

The total number of patients investigated by this method was 47. This number is smaller than the total number of patients investigated by histological methods because this part of the project was begun later, and because material was discarded for a variety of reasons. Tissue

was discarded if it did not reach the laboratory within an hour of its removal. A total of 21 specimens was excluded from the series because the final preparation contained too few epithelial cells for an adequate count to be made.

In addition, three specimens were not included in the series because the diagnosis on the original breast lump was carcinoma. Three other specimens were taken from patients who proved to be post-menopausal.

Age

The age range was 15 - 48, with a mean of 29.8. All patients were menstruating, and all were Caucasian.

Diagnosis

The diagnoses of the breast lumps biopsied are set out in Table 5:1. The diagnoses are divided into two groups: Group 1, in which there was either no breast disease or a localised lump such as a fibroadenoma; and Group 2, in which the disease, though benign, might not have been localised to the lump excised. However, all the tissue examined in this study was histologically normal.

Parity and Stage of the cycle

The parity and stage of the cycle of the patients studied are set out in Table 5:II. The history was obtained as described in Chapter 4. Blood was obtained for estimation of concentration of progesterone and oestradiol-17β, as described in Chapter 4.

TABLE 5:I Histopathology of the primary condition

Group 1:	Fibroadenoma	12
(22 women)	Reduction mamoplasty	5
	Chronic inflammation	1
	Normal tissue	1
	Lymph node	1
	Dilated duct	1
	Abscess	1
Group 2:	Fibroadenosis	17
(25 women)	Fibrocystic disease	6
	Lobular adenosis	1
	"Mixed" benign picture	1
TOTAL		47

TABLE 5:II Parity and Stage of the cycle

Nulliparae	- normally cycling	13
	- on oral contraceptive	3
Parous women	- normally cycling	24
	- on oral contraceptive	7
TOTAL		47

C METHOD

The method of removing specimens in the operating theatre was identical to that described in Chapter 4.

Immediately after excision, specimens approximately 5 x 3 x 2 mm in size were transferred into Waymouth's MB 752/1 medium (Flow Laboratories, Irvine), containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N^1-2-ethanesulphonic acid) (Flow Laboratories). Within an hour of excision, the tissue was cut into 1 mm slices using paired razor blades, and transferred to 5 ml of fresh medium (pH 7.2) containing 2μCi methyl-^{3}H-thymidine / ml (Radiochemical Centre, Amersham; 5 Ci/mmol.) in a sealed plastic Universal container. After four hours' incubation at 37^{o}C, the tissue was washed three times in Hanks' balanced salt solution and fixed in formol saline.

The tissue was processed by routine histological procedures, and 5μ paraffin sections were picked up on "subbed" slides. Autoradiographs were prepared using Kodak AR10 stripping film, and exposed for 14 days at 4^{o}C. The autoradiographs were developed for 5 minutes in Kodak D19 developer, and fixed for 10 minutes in Ilford Hypam: distilled water (1:5): both processes were carried out at 17^{o}C. The sections were washed, dried, stained with haematoxylin and eosin, and mounted in DPX.

At least 1000 epithelial cell nuclei were counted in each specimen (average count 1,446), and the number of labelled nuclei was noted: any nucleus with more than five grains was scored as positive. The Labelling Index (LI) was expressed as the number of labelled nuclei per 1000 after four hours' incubation with tritiated thymidine. Results were statistically analysed by the Wilcoxon Rank Sum Test.

D RESULTS

1 Repeatability

The repeatability of the observations was tested with tissue from four patients. (Attempts to compare right and left breasts in three other patients were unsuccessful because no epithelium was present in one of the samples from each patient).

So as to eliminate observer bias, the specimens were labelled in such a way that they did not appear to come from the same patient.

The results are given in Table 5:III below.

TABLE 5:III Repeatability of observations

Patient number	Areas sampled	Labelling Indices
JD 74	Right breast	3.0
	Right breast	1.9
JD 110	Left upper inner quad	20.0
	" " " "	22.2
JD 163	Right breast	4.2
	Right breast	3.7
	Right breast	4.6
JD 168	Left breast	3.0
	Right breast	1.8

The correlation coefficient between samples is 0.998

2 Diagnosis

Patients were divided by diagnosis in the lump biopsied, as shown in Table 5:1. The mean LI in Group 1 (patients with localised disease only) was 6.92 ± 1.61. The mean LI in Group 2 (patients whose disease may not have been localised) was 9.44 ± 1.40. The difference between these groups is not statistically significant.

3 Parity

Among the 17 nulliparous women the mean LI was 8.09 ± 1.94. This was not significantly different from the mean LI among the 31 parous women, which was 8.39 ± 1.28. The parity of the parous women ranged from 1 + 0 to 4 + 2, and the number of pregnancies a woman had had did not effect the LI (Fig 5:1).

4 Stage of the menstrual cycle

The labelling indices were compared with the day of the cycle on which the biopsy was taken, in both nulliparous and parous women. In Figs 5:2 and 5:3 the day of cycle is taken as the number of days from the onset of the last period, as recalled by the patient. Among the 14 nulliparous patients there was no sign of a cyclical pattern (Fig 5:2), and there was no significant difference between mean LI's in the two phases of the cycle (Table 5:IV).

Among the 31 parous patients, however, there was a biphasic pattern (Fig 5:3). The Labelling Indices fell steadily after the start of the cycle to a low point at the time of ovulation, and during luteal phase they rose again to a maximum just before menstruation. The mean LI during the follicular phase among parous women was 5.38 ± 1.63, which is significantly different from the LI during the luteal phase (12.17 ± 2.21). ($P<0.05$ Table 5:IV).

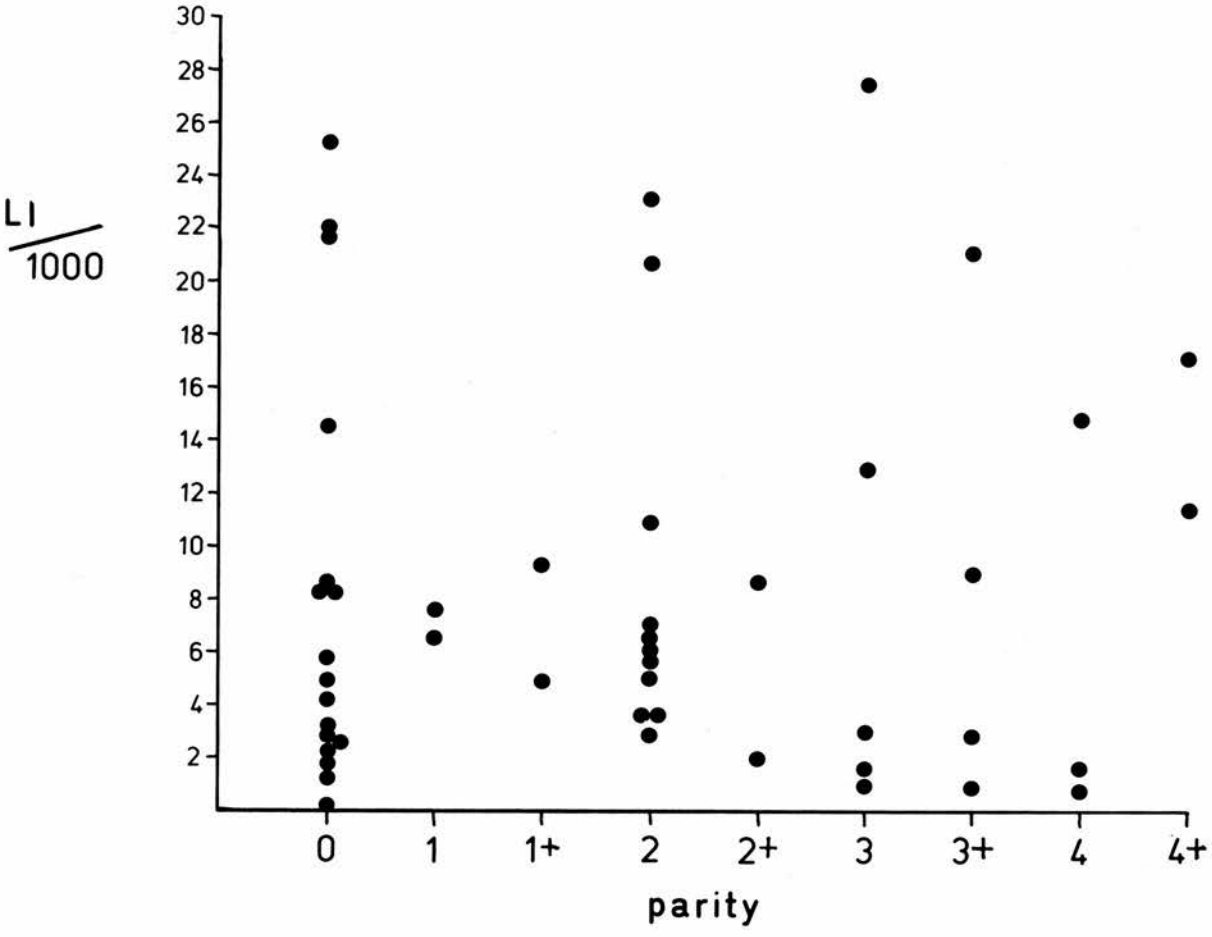


Fig 5:1 Relationship between epithelial cell Labelling Index (LI) and parity. The figures for parity represent completed pregnancies of more than 28 weeks' gestation. "+" means that the patient had also had one or more miscarriages.

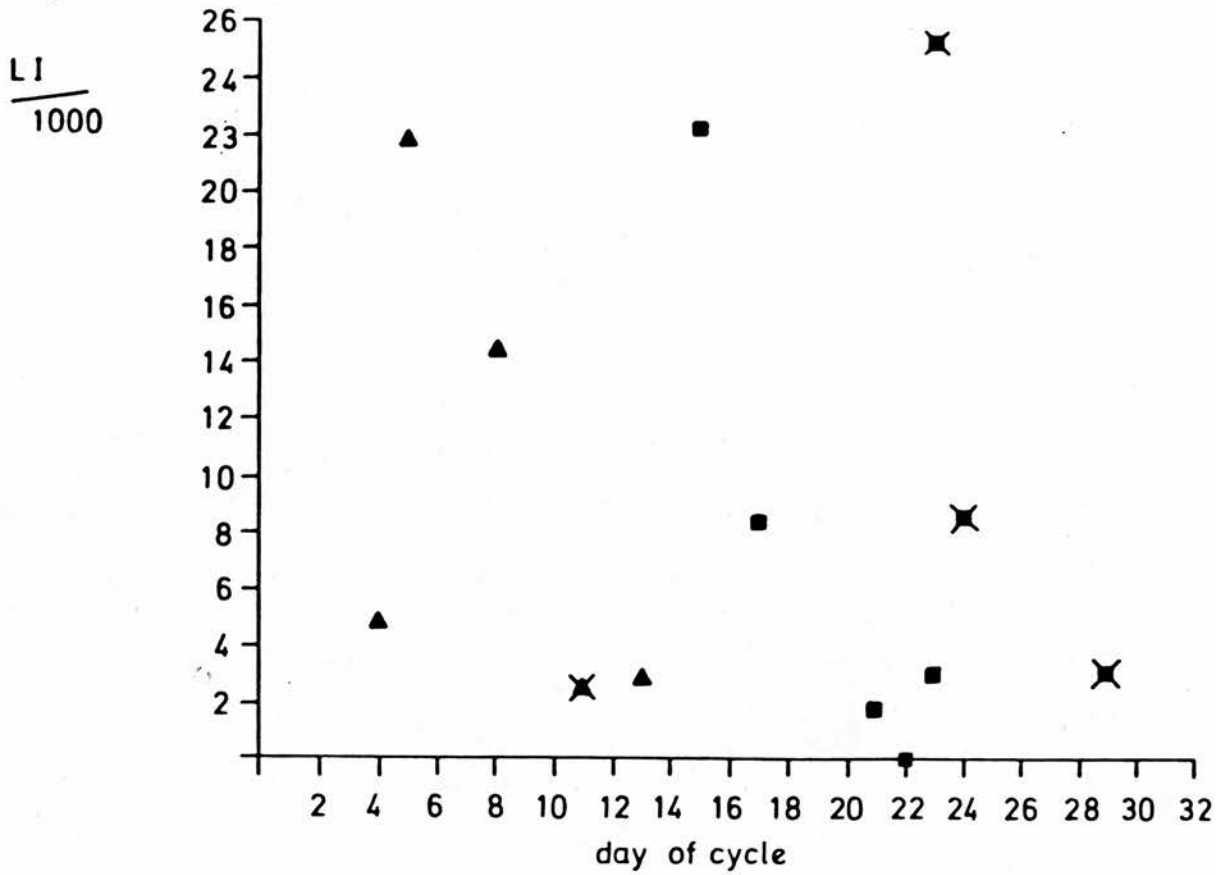


Fig 5:2 LI v day of cycle in nulliparae.
Squares denote subjects with plasma progesterone greater than Ing/ml (3.2 nmol/L).
Crosses denote specimens obtained at mammoplasty.

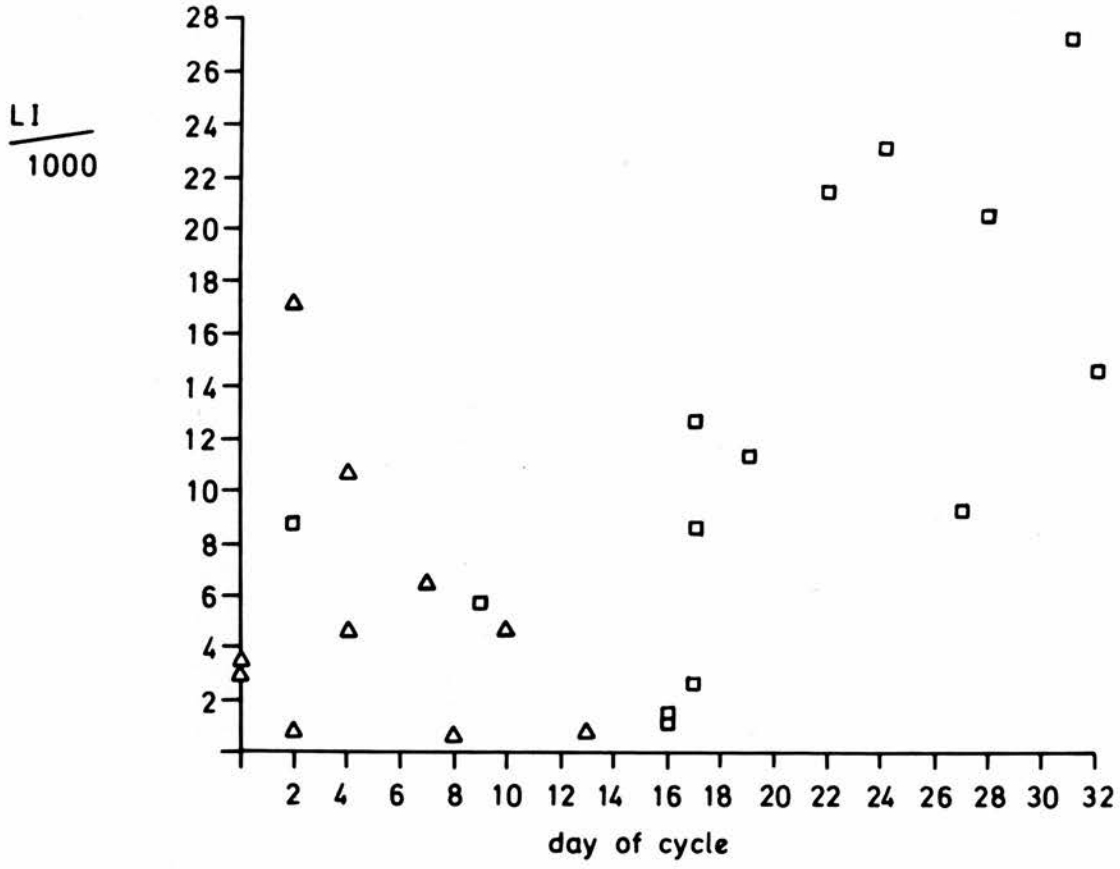


Fig 5:3 LI v day of cycle in parous women.
Squares denote patients with plasma progesterone greater than Ing/ml (3.2 nmol/L).

Because of variation in the length of the cycle the simple method of dating the cycle used in Fig 5:2 and Fig 5:3 may not be an accurate reflection of the influence of ovulation and the luteal phase. In addition, the patient's recollection of the date of her last period may be inaccurate. For these reasons, Labelling Indices were plotted against the number of days until the next period (as notified to me by letter from the patient). The resulting "back plot" is shown in Fig 5:4.

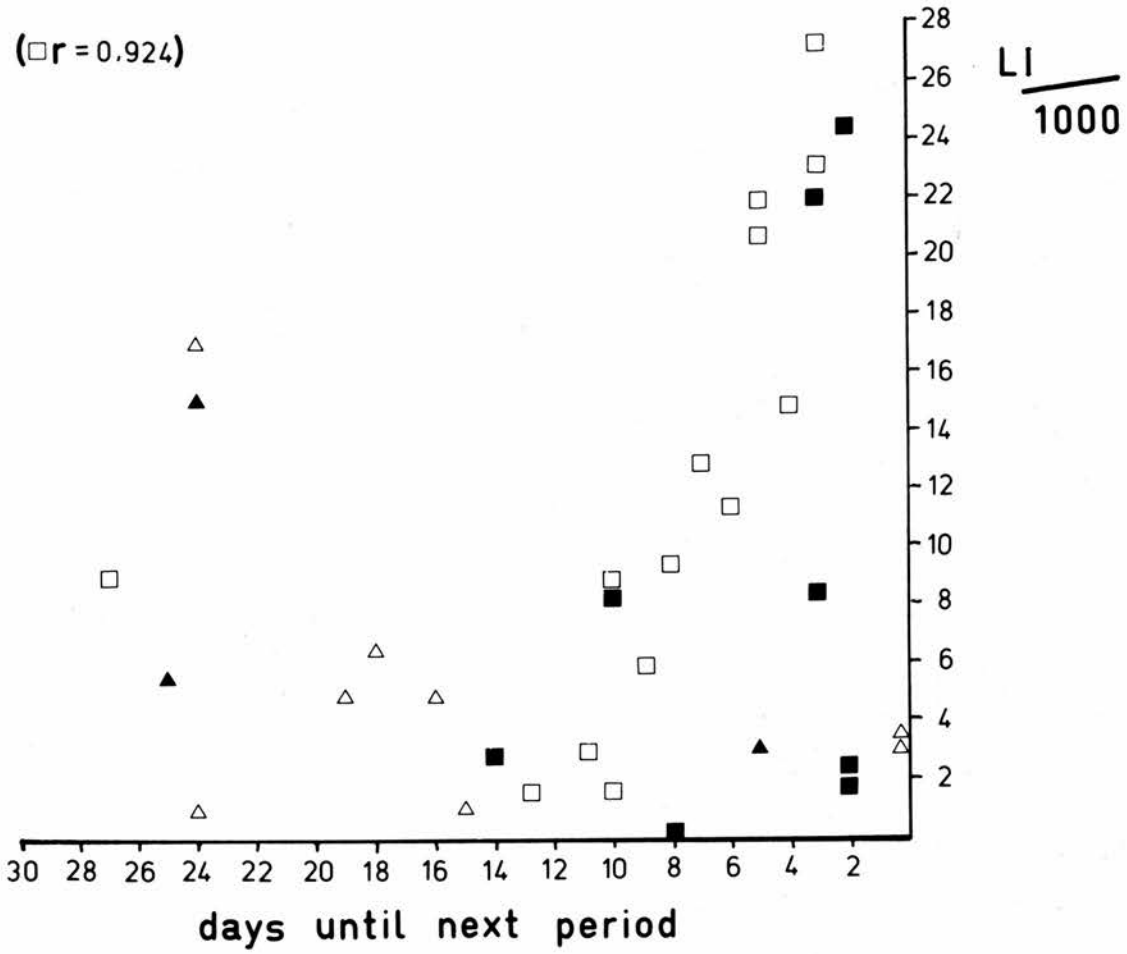
In Fig 5:4 the parous women show a similar biphasic curve to that seen in Fig 5:3. However, the most striking feature of the "back plot" is the steady rise in LI among parous women during the luteal phase - ie. from 13 days before the next period until three days before the next period. Among parous women the correlation between LI and the day of the luteal phase (dated from the onset of the next period) is 0.924. This correlation is highly significant ($P<0.001$).

Among nulliparae no biphasic curve is seen in the "back plot", and the correlation with the day of the luteal phase is not significant ($r:0.381$).

5 Plasma progesterone and oestradiol 17β

Plasma progesterone concentration in the blood sample taken at the time of operation was compared with LI in both nulliparous and parous women. Among nulliparae there was no correlation (r: 0.587). Among parous women, (surprisingly perhaps, in view of the relationship seen in Fig 5:4) there was no correlation, with a correlation coefficient of 0.077 (Fig 5:5)

Oestradiol 17β showed no correlation with LI in either nulliparae or parous women (r: 0.409 and 0.113 respectively) (Fig 5:6)



▲ Nulliparous: plasma progesterone below 1 ng/ml

△ Parous: plasma progesterone below 1 ng/ml

■ Nulliparous: plasma progesterone greater than 1 ng/ml

□ Parous: plasma progesterone greater than 1 ng/ml

Fig 5:4 Epithelial cell Labelling Index (LI) back-plotted from the first day of the subsequent period, to show the effect of the luteal phase on LI. Between Day 14 and Day 0 LI rises steadily among parous women, and the correlation coefficient for this sub-group is shown on the figure.

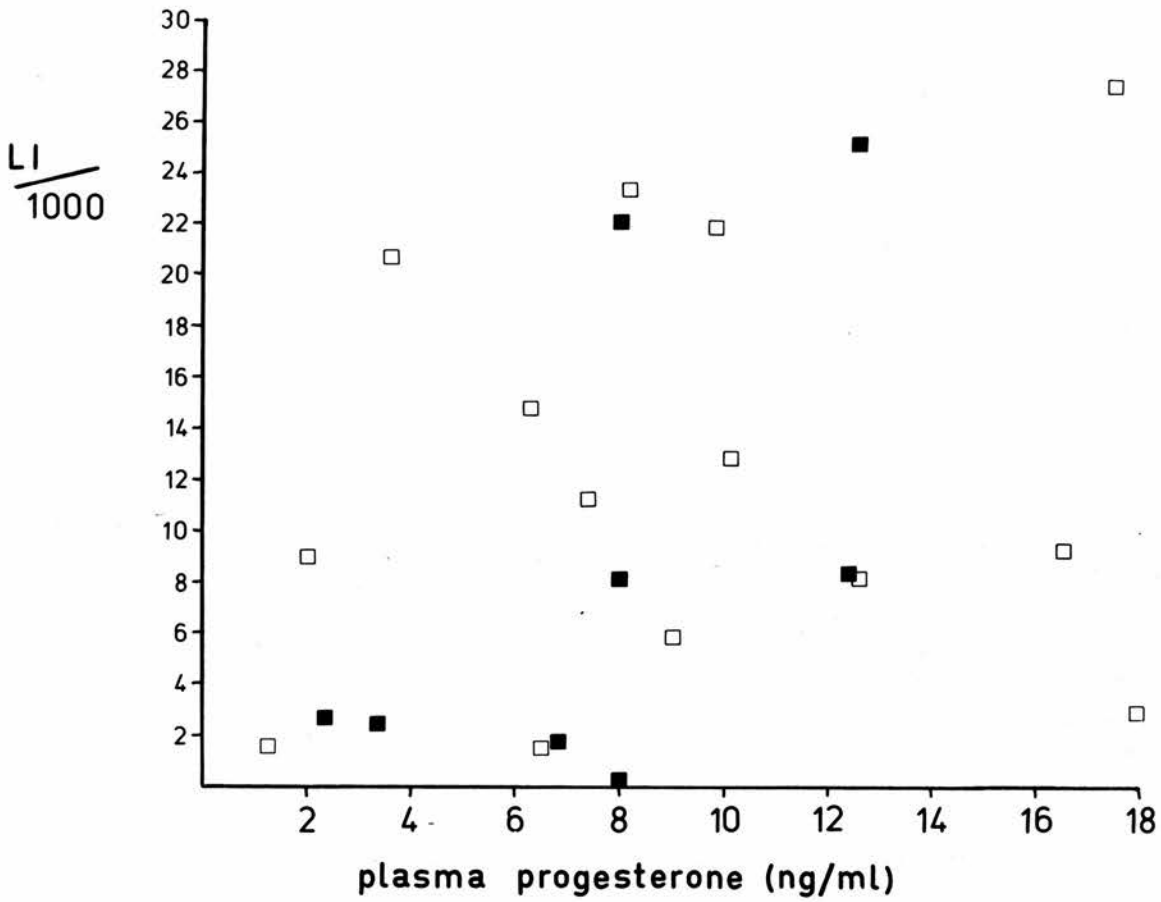
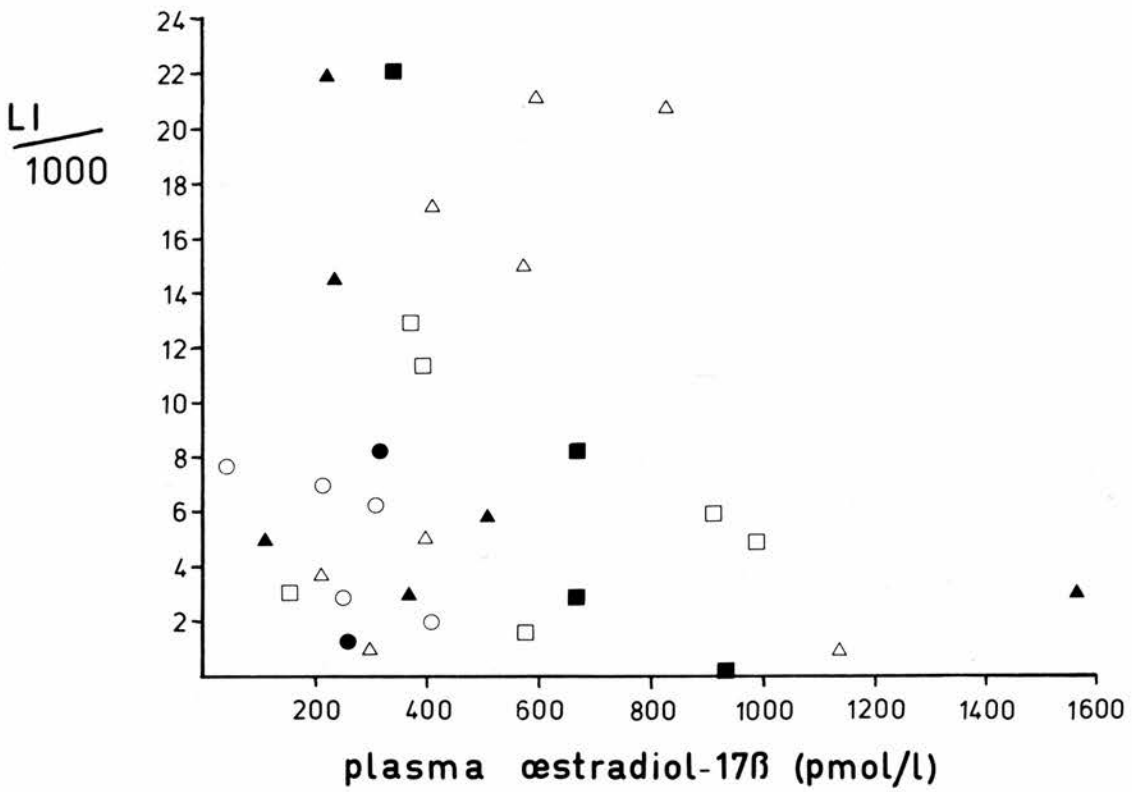


Fig 5:5 Relationship between epithelial cell Labelling Index (LI) and plasma progesterone concentration. There is no significant correlation in either parous or nulliparous women (see text).



▲ Nulliparous: plasma progesterone below 1 ng/ml

△ Parous: plasma progesterone below 1 ng/ml

■ Nulliparous: plasma progesterone above 1 ng/ml

□ Parous: plasma progesterone above 1 ng/ml

● Nulliparous: taking oral contraceptive

○ Parous: taking oral contraceptive

Fig 5:6 Relationship between epithelial cell Labelling Index (LI) and plasma oestradiol-17β concentration. There is no significant correlation in either parous or nulliparous women (see text).

TABLE 5:IV Labelling Indices:

Influence of parity, stage of the menstrual cycle, and oral contraception

NULLIPARAE			PAROUS WOMEN		
On Pill	Prolif phase	Luteal phase	On Pill	Prolif phase	Luteal phase
(n:3)	(n:6)	(n:8)	(n:7)	(n:10)	(n:14)
4.57	8.76	8.90	5.15	5.38*	12.17*
±2.04	±3.17	±3.40	±0.86	±1.63	±2.21

ALL Nulliparae: 8.09 ± 1.93 ALL Parous: 8.39 ± 1.28

* The difference between the proliferative and luteal phases among parous women is significnat ($P<0.05$)

TABLE 5:V Labelling Indices:

Influence of breast feeding history among parous women

Never breast-fed	DURATION OF BREAST FEEDING				All breast-feeders
	1-6 wks	8-20 wks	24-36 wks	52-84 wks	
(n:14)	(n:4)	(n:5)	(n:4)	(n:4)	(n:17)
7.44	11.62	5.16	5.27	10.70	8.00
±1.75	±3.4	±3.02	±2.00	±4.50	±1.67

6 Oral Contraception

Seven parous women and three nulliparae were taking the oral contraceptive pill during the month in which the biopsy was performed. Their LI's are in the same range as those of non-Pill users, and are in the lower part of this range: they do not differ significantly from those in normally cycling women (Table 5:IV). The LI's are plotted against the day of the cycle in Fig 5:7. No relationship can be seen to the day of the cycle, although the numbers of specimens examined are small.

7 Menstrual Age (ie. Age minus age at menarche)

The menstrual age of the patients is compared with the LI in Fig 5:8. There is no correlation in either nulliparous or parous women.

8 Birth Interval (ie. Age at first birth minus age at menarche)

The number of years between a parous woman's menarche and her first full-term pregnancy was compared with her LI. The results are presented in Fig 5:9. There is a suggestion of a negative correlation. Among women in the follicular phase of the cycle there is no obvious relationship, but among women in the luteal phase there is a more obvious negative correlation, with lower LI's being found among women who had a later first pregnancy. Over all parous women the relationship is a significant one, the correlation coefficient between LI and birth interval being 0.531 ($P<0.01$).

This relationship was analysed further, and a check was first made to find out whether a coincidental relationship existed between the birth interval and the stage of the cycle on which the biopsy was taken. Fig 5:10 illustrates that there is a clear relationship between the birth interval and the number of days until the next period, among parous women

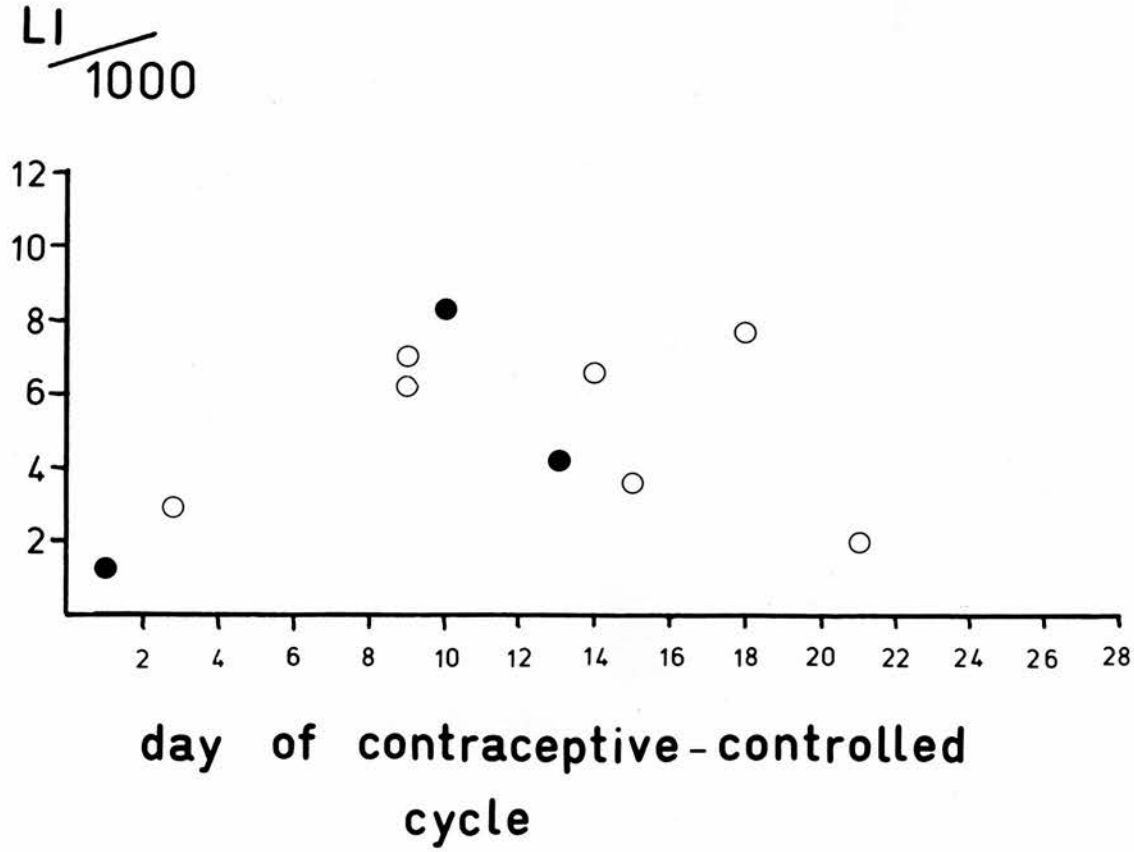


Fig 5:7 Epithelial cell Labelling Index (LI) plotted against day of oral contraceptive-controlled cycle.

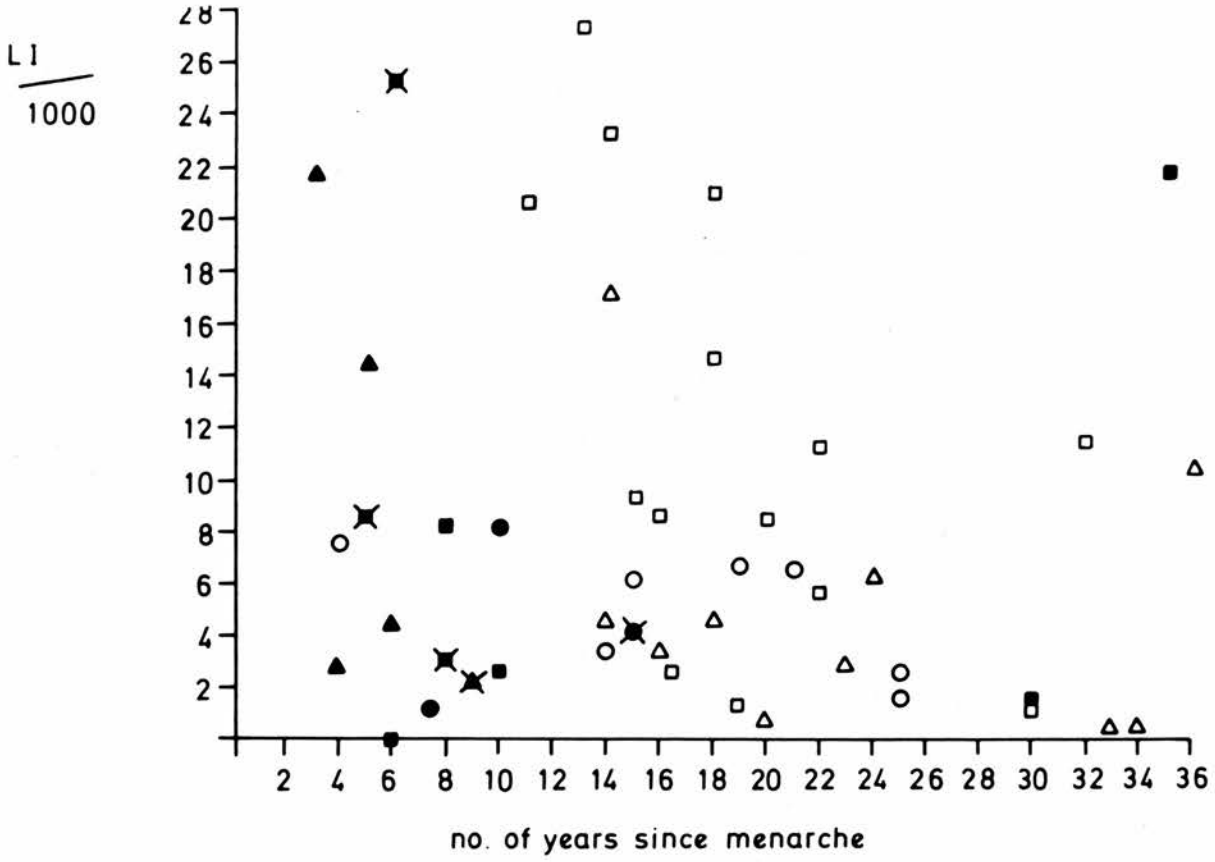


Fig 5:8 LI v menstrual age

Open symbols - parous subjects

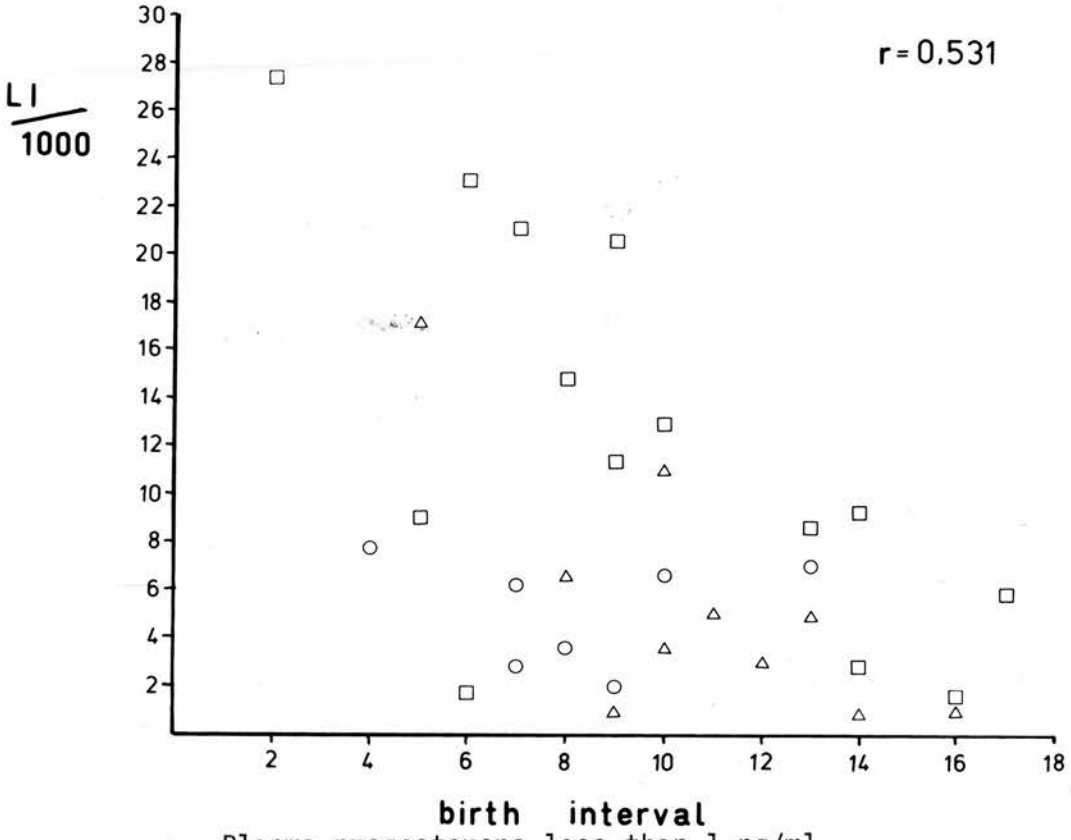
Closed symbols - nulliparous subjects

Squares - plasma progesterone greater than 1 ng/ml (3.2 nmol/L)

Circles - Pill users

Crosses - mammoplasty specimens

Triangles - plasma progesterone less than 1 ng/ml



△ Plasma progesterone less than 1 ng/ml

□ Plasma progesterone greater than 1 ng/ml

○ Taking oral contraceptive

Fig 5:9 Relationship between epithelial cell Labelling Index (LI) and "birth interval" - ie. the number of years between the menarche and the first full-term pregnancy.
Overall there is a significant negative correlation, but see text and Fig 5:10.

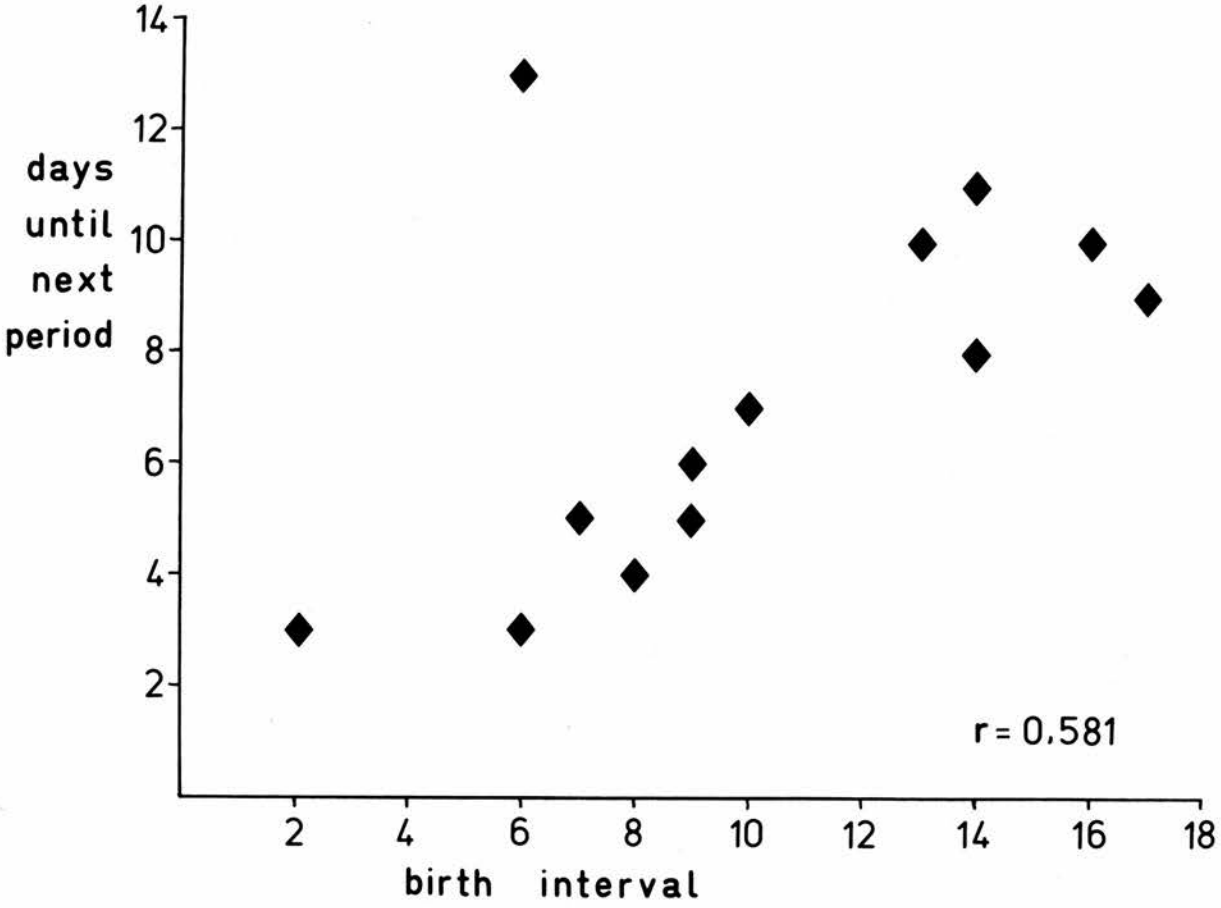


Fig 5:10 Parous women in the luteal phase of the cycle. Among the patients studied there was a relationship between the day of the luteal phase (plotted as "number of days until next period") and the birth interval - ie. the number of years between the menarche and the first full-term pregnancy. This has to be taken into account in interpreting the relationship shown in the previous figure (Fig 5:9).

in the luteal phase of the cycle (r: 0.581: P<0.05). This relationship appears to be entirely coincidental, but it complicates the analysis of the data. This is discussed further in the final section of this chapter.

9 Breast Feeding History (Table 5:V)

Of the 31 parous patients, 14 had never breast-fed. The total duration of breast-feeding (adding up all the pregnancies) in the remaining 17 women ranged from two weeks to 21 months. Table 5:V shows the effect of the duration of breast-feeding by dividing breast-feeders into four groups depending on the total duration of lactation. There are no significant differences between these four groups, and there is no difference between breast-feeders and non-breast feeders overall.

DISCUSSION

The labelling index (LI) is an indicator of the level of DNA synthesis, and indirectly of cell growth. Specific conclusions about cell growth rates cannot be drawn from the LI alone, but in general an increase in the number of labelled cells indicates an increase in cell growth.

Munford (1964), in a study of various quantitative methods of assessing changes in the mammary gland, concluded that changes in DNA are an indication of histological changes, and that biochemical changes occurred in the absence of histological changes.

Flaxman and Lasfargues (1973) also used tritiated thymidine incorporation and autoradiography, but used a different method (Flaxman and van Scott 1972) of in vitro culture of human breast epithelial cells, which involves the addition of hormones. Flaxman and Lasfargues showed that DNA synthesis would occur in vitro in epithelial cells of human mammary gland without

added hormones, but addition of insulin and prolactin caused an increase in DNA synthesis. No other hormones were tested. In our technique, no hormones were added, and the changes in LI were due to changes already brought about by circulating hormone.

Ceriani (1972) also maintained normal human breast tissue in organ culture: he reported that addition of oestrogen caused lobuloalveolar development, as did addition of progesterone. Progesterone stimulation produced small empty alveoli, and oestrogen stimulation produced large alveoli with secretion. These effects were enhanced if prolactin and insulin were added to the medium.

Our results show an increase in LI during the luteal phase among parous women, with a steady decrease among these women during the follicular phase. Among nulliparae, no consistent change could be seen: although the numbers of nulliparae were small, the scatter of results seems to suggest that the same cyclical pattern does not occur among nulliparae.

Meyer (1977) carried out a similar study to ours, at the same time as ours. Although he examined 104 specimens, only 49 of these were from menstruating women in whom the stage of the cycle was known. The stage of the cycle was assessed by history alone, and no distinction was made between nulliparous and parous women. Meyer reports that a diurnal variation has been noted in LI among experimental animals, and that this was minimised in his study since almost all specimens were obtained between 8 am and noon. (The same is true for our study, although the hours were 9 am and 1 pm). Meyer found that the LI was low between days 2 and 15 of the menstrual cycle, and "often elevated" between days 16 and 1. His results are identical to the results we obtained among parous women (Fig 4:7): he includes six patients taking oral contraceptives

and they appear to follow the same cycle. The parity of his subjects is not reported. Fibroadenoma tissue appeared to follow a similar cycle. Two samples from patients in the middle trimester of pregnancy showed LI's at the upper end of the normal range. He also reports a negative correlation with age.

In our results, when the LI's among parous women are back-plotted according to the first day of the subsequent period, a linear relationship is seen, but when the LI's are plotted against the progesterone concentration, there is no correlation. This suggests that the duration of exposure to progesterone is more important than the progesterone concentration. If this is so, it implies that there is a "threshold level" of progesterone in the circulation that stimulates the epithelial cells, rather than a graded response.

It would be interesting to know whether or not the plasma concentration of progestogen produced by the combined oral contraceptive pill is above such a postulated "threshold" level. Unfortunately, after exclusions the number of patients examined was insufficient to allow conclusions to be drawn. Graph 5:8 suggests that only 10-14 days' exposure to progestogen is sufficient to raise the LI above 20, while Graph 5:9 suggests that among the few parous women who had been taking the pill for 9-14 days before their biopsy the LI had not reached a high level (varying from 2 to 8). Although this suggests that the oral contraceptive pill does not contain enough progestogen to stimulate the epithelial cells, the numbers of specimens are too small for this to be a firm conclusion.

The correlation between the number of years of nulliparity and the day of the luteal phase on which the biopsy was taken is an unfortunate

coincidence. There cannot possibly be a cause-and-effect relationship between these two variables, but the fact that they are related in this series means that one cannot be sure about which is the important variable governing the amount of DNA synthesis. DNA synthesis correlates equally well with each. If in fact the important variable is the number of years of nulliparity, this would imply that the longer the breast goes before first pregnancy, the less is its ability to respond to progesterone: one might expect this to be reflected clinically in poorer breast-feeding among older women, and perhaps even in an inability to breast-feed beyond a certain age. Since this does not happen it is perhaps more likely that the important variable is the stage of the luteal phase. The uncertainty could have been resolved in our series by obtaining tissue for examination late in the luteal phase from women who had delayed their first pregnancy until their thirties - but such patients proved difficult to find.

SUMMARY

DNA synthesis in vitro was studied in specimens of breast tissue from 47 women, by organ culture with tritiated thymidine followed by autoradiography. Among parous women cyclical variation of labelling was seen, with highest values during the luteal phase. No cyclical change was seen among nulliparae. Labelling was not related to plasma concentrations of progesterone or oestradiol 17B, nor to menstrual age or breast-feeding history. A negative correlation with birth interval was noted, which may have been due to a coincidental correlation between birth interval and stage of the cycle.

CHAPTER SIX

IMMUNOLOGICAL STUDIES

I INTRODUCTION

During lactation the breast secretes large quantities of immunoglobulins, but very little is known about the production of immunoglobulins by the non-lactating breast. An abstract (Hochwald et al 1964) included "breast" as one of the tissues which produced B_2A globulin in vitro, but otherwise the normal non-lactating gland has not been examined for immunoglobulin synthesis. Part of the reason, it has been suggested (Hurliman et al 1976), has been difficulty in culturing the tissue.

Techniques are now well advanced for the identification of immunoglobulins, and they can be studied in a semi-quantitative way. It is known that milk contains IgA, IgM and IgG, and that colostrum is particularly rich in immunoglobulins. In early human colostrum the concentration of total immunoglobulins is 4-5 Gm%, almost 90% being IgA and IgM (Hanson and Johanson 1970). (The main circulating immunoglobulin, by contrast, is IgG).

A system for the identification of immunoglobulins in intestinal mucosa had been developed by Dr D B L McClelland of the Department of Therapeutics of the University of Edinburgh. For the reasons given above, it was felt that the adaptation of this system for the study of normal breast tissue would be worthwhile. I am most grateful for the help of Dr McClelland and his colleagues (acknowledged in the introduction to this thesis).

II BIOPSY TECHNIQUE

The method of obtaining tissue was identical to that described in Chapter 4. Apparently normal tissue (as judged by the surgeon) was removed via the same skin incision as the breast lump being biopsied, but as far away from the obviously abnormal tissue as possible. The tissue was transported to the laboratory in normal saline, and was processed within 1½ hours of its being removed from the patient.

III HISTOLOGY OF THE TISSUE STUDIED

Sections cut serially with those used for immunofluorescence (see below) were stained with haematoxylin and eosin and examined by two observers, including a pathologist who had no knowledge of the patients. Any specimens in which pathological lesions were found were excluded from the study. Sections from the adjacent formalin-fixed blocks were also examined, and no pathological features were seen, though a few of these sections showed minor changes (atrophic changes in five specimens, small cysts in two, ductular dilatation in seven, and features of fibrosing adenosis in three). No specimens showed epitheliosis.

IV IN VITRO SYNTHESIS OF IMMUNOGLOBULINS

A) Patients studied

80 specimens were taken from 74 women, all of whom were experiencing regular menstrual cycles. The age range was 15-52, with a mean of 33. 27 were nulliparous and 47 had had at least one full-term pregnancy. Of these, seven nulliparous subjects and six parous subjects were taking the oral contraceptive pill at the time of the biopsy.

A further 20 specimens in addition to the 74 were discarded from the series - either because the breast lump biopsied turned out to be malignant,

or because the tissue removed for study turned out to be abnormal.

As already described, a full reproductive history was taken from each patient. She notified me of the date of her subsequent period by letter. Blood was taken, either at the time of operation or within 24 hours thereafter, for estimation of plasma progesterone and oestradiol-17β concentrations.

B) Histopathology of the primary condition

In all cases the lesion biopsied proved to be benign breast disease. (If the lesion proved to be malignant, the patient was excluded from the study). The primary diagnosis of all the specimens was checked by Dr I I Smith of the Department of Pathology, University of Edinburgh.

The diagnosis is shown in Table 6:1.

The patients were divided into two groups. Group 1 consists of cases in which the disease would be expected to be confined to the excised lump (eg. fibroadenoma) or in which no disease was present (eg. reduction mammoplasty specimens): in this group the rest of the breast could be expected to consist of normal tissue. In Group 2 the diagnosis was fibrosing adenosis, a term which covers both fibroadenosis and fibrocystic disease: this condition may not necessarily have been localised to the excised lump, even though the tissue used in this study was histologically normal.

TABLE 6:1a

Histopathology of the primary condition (in vitro synthesis)

Group 1	Fibroadenoma	42 specimens from 40 women
	Reduction mammoplasty	20 specimens from 19 women
	No abnormality found	7 specimens from 7 women
	Lipoma	1 specimen from 1 woman
Group 2	Fibrosing adenosis (with or without cyst formation)	42 specimens from 40 women
TOTAL		80 specimens from 74 women

TABLE 6:1b

Histopathology of the primary condition (immunofluorescence)

Group 1	Fibroadenoma	8 specimens from 7 women
	Reduction mammoplasty	4 specimens from 2 women
	No abnormality found	3 specimens from 3 women
	Lipoma	1 specimen from 1 woman
Group 2	Fibrosing adenosis (with or without cyst formation)	18 specimens from 18 women
TOTAL		34 specimens from 31 women

C) Immunological Methods

80 specimens in all, each weighing 100-150 mg, were finely chopped with scalpels and cultured for 48 hours at 37°C in a roller tube, following the method detailed by van Furth et al (1966) and McClelland et al (1976). The culture medium was 1 ml of modified Eagle's medium containing 1 μCi/ml [^{14}C] L-lysine and 1 μCi/ml [^{14}C] L-isoleucine (specific activity more than 270 μCi/mmol: Radiochemical Centre, Amersham). The medium contained gentamicin (25 μg/ml) and nystatin (75 μg/ml). After incubation the cultures were frozen and thawed three times, centrifuged, and the supernatants dialysed against two changes of phosphate buffer (0.015M: pH 7.6) to remove excess labelled amino acids. The culture fluids were then freeze-dried and resuspended in 0.1 ml of distilled water.

1) Immunoelectrophoretic analysis of the culture fluid was performed using 6μL of the concentrated culture fluid. Because this fluid contains too little protein to provide good reproducible precipitation lines, a serum carrier was used. The antigen well was filled once with the carrier serum. After the serum had penetrated into the agar (3-4 minutes), the same antigen well was filled three times with the concentrated culture fluid. After electrophoretic separation, the antiserum trough, at 5 mm from the antigen well, was filled with equine antiserum against human serum, or other antisera - see below. The precipitation lines were developed for 24 hours at room temperature. The slides were washed for 72 hours in phosphate-buffered saline, dried, and stained with 0.5% Amino Black 10B (Merck).

2) Autoradiography was performed at room temperature with a sheet film (Kodak Royal-X pan) cut into strips to fit the microscope slides. The exposure time was 21 days.

During development of the precipitation lines the labelled proteins from the culture fluid and the proteins from the carrier serum precipitate together. Labelling of an immunoglobulin line indicates the synthesis of this protein in vitro. (See Fig 6:1)

3) Evaluation of the intensity of protein synthesis in vitro

The intensity of the autoradiographic lines, which indicates the amount of protein synthesised (van Furth et al, 1966: Lai A Fat et al, 1976), was classified according to a scale ranging from:

-	negative
±	line barely visible
+	line clearly visible

to a maximum of +++ - a very dark line on the autoradiograph. The grading was done independently by two observers.

4) Antisera Antiserum to whole human serum (raised in the horse) and antiserum to IgM (raised in rabbits) were obtained from the Central Laboratory of the Red Cross Blood Transfusion Service, Amsterdam. Commercial antisera to human IgA were obtained from Hoechst Pharmaceuticals Ltd, Brentford, Middlesex (raised in rabbits), and from Nordic Laboratories, Tilburg, the Netherlands (raised in goats). Antiserum against human serum IgA, provided by Dr J Radl, Institute for Experimental Gerontology, TNO, Rijswick, the Netherlands, was raised in rabbits against a pool of human serum IgA proteins and purified by solid phase immunoabsorption (McClelland et al, 1976). Neither this antiserum nor the two commercial anti-IgA sera showed any reaction with secretory component.

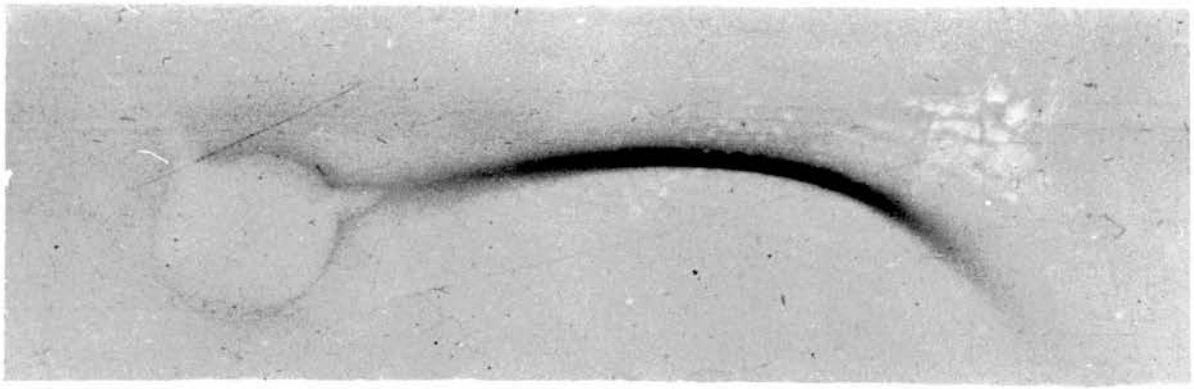


Fig 6:1 Immunoglobulin synthesis in vitro detected by radioimmunoelectrophoresis.

Above: Stained immunoelectrophoresis pattern developed with anti-IgA serum showing IgA arc. Carrier is human milk.

Below Autoradiograph showing labelling if IgA arc.

The antiserum to human secretory component was kindly provided by Dr P J J van Munster, Department of Paediatrics, University of Nijmegen, the Netherlands, and its characteristics have been reported in detail (van Munster et al 1969). This antiserum reacts with free secretory component and also with an antigenic determinant which is specific to secretory component bound to IgA. It does not react with serum IgA or IgA polymers.

D) Plasma cells

Sections stained with Methyl Green Pyronin (Drury and Wallington 1967, p 160) were examined for the presence of plasma cells, identified by their characteristic shape and cherry-red cytoplasm. Counts were made of the number of cells per high-power field within lobules. A minimum of three fields per specimen was examined, and the mean count was obtained.

E) Results

Synthesis of IgA was detected in 81% of the 80 specimens and synthesis of IgG in 45% (Table 6:2). IgA synthesis was "strong" (++ or +++) in 42% of the positive specimens, whereas 94% of the IgG-positive specimens showed only weak or barely detectable synthesis (+ or ±). IgM synthesis was seen in two specimens, and was barely detectable in both.

1) Repeatability of results Four specimens were taken from different parts of a single breast at mammoplasty, and coded as separate specimens: none of the observers knew which specimens came from the same patient. After organ culture IgA synthesis was graded as +++ in two and ++ in the other two; IgG synthesis as ++ in two and + in two; and IgM as negative in all four.

2) Influence of parity and stage of the menstrual cycle (Table 6:3)

When comparison was made between parous and nulliparous women, the proportions of positive specimens (81% and 77%) were similar. There was no significant difference between the tissue removed during the luteal phase of the cycle (as shown by a plasma progesterone concentration greater than 1 ng/ml (3.2 nmol/l)), and that removed in the remainder of the cycle. However, when the influence of the stage of the cycle was examined in parous and nulliparous women separately, cyclical changes were seen in the parous women but not among the nulliparae (Table 6:3).

Among parous women, 16 showed strong (++ or +++) IgA synthesis during the luteal phase, compared with only three in the proliferative phase ($P<0.05$). Among nulliparae there was no significant difference between the phase of the cycle. When patients in the luteal phase of the cycle were examined, IgA synthesis was more intense among parous women than among nulliparae, and the difference was highly significant ($P<0.005$).

The pattern of IgA synthesis was similar among women taking oral contraceptives to that among normally cycling women. 13 women were taking oral contraceptives (one parous woman taking oral contraceptives provided two specimens of tissue)and IgA synthesis was "strong" (++) in three specimens, and "weak" (+ or $\pm$) in ten specimens.

The relationship of intensity of IgA synthesis to plasma progesterone concentration and plasma oestradiol concentration is shown in Tables 6:4 and 6:5. There does not appear to be a relationship to the concentration of either hormone in nulliparous or in parous women.

TABLE 6:2

Synthesis of immunoglobulins by breast tissue cultured in vitro

	Number of positive specimens (and % of total)	Mean intensity of labelling of autoradiographs (number of specimens)			
		+++	++	+	±
Total (80 specimens)					
IgA	65 (81%)	9	18	24	14
IgG	36 (45%)	-	2	14	20
IgM	2 (3%)	-	-	-	2

TABLE 6:3

Influence of parity and stage of the menstrual cycle on in vitro synthesis of IgA

	Phase of the menstrual cycle	Number of positive specimens (and % of total)	Mean intensity of labelling of autoradiographs (number of specimens)			
			+++	++	+	±
Nulliparous patients (Total 30 specimens)	Follicular (Total 12)	11 (92%)	2	2	7	-
	Luteal (Total 11)	6 (55%)	-	1	4	1
	Oral contraceptive (Total 7)	7 (100%)	-	1	2	4
Parous patients (Total 50 specimens)	Follicular (Total 17)	12 (71%)	1	2	5	4
	Luteal (Total 26)	23 (88%)	6	10	3	4
	Oral contraceptive (Total 7)	6 (86%)	-	2	3	1

4) Influence of diagnosis of the primary condition Table 6:6 shows that the number of positive specimens and the pattern of immunoglobulin synthesis was similar in Group 1 and Group 2. The proportion of specimens showing synthesis was greater in Group 2, but the difference was not significant (X^2 : 0.57; $P>0.1$).

5) Other Factors As in Chapters 4 and 5, comparisons were made between menstrual age (Table 6:7), birth interval (Table 6:8) and breast-feeding history (Table 6:9) and IgA synthesis. The tables show that none of these factors appeared to have an influence on IgA synthesis.

6) Correlation with numbers of plasma cells When adjacent sections stained with Methyl Green Pyronin were examined, it was found that in all but two specimens plasma cells were present in larger numbers within lobules than in the extralobular stroma. Among specimens in which IgA synthesis had been detected in organ culture the mean number of plasma cells per high-power field within lobules was 5.4 ($\pm$ 4.3). Among specimens in which no IgA synthesis had been detected in organ culture, the mean number of plasma cells per high-power field within lobules was 2.2 ($\pm$ 1.9) The difference between these groups is significant ($P<0.05$). However, the numbers of plasma cells showed no correlation with the intensity of synthesis among specimens showing IgA synthesis.

TABLE 6:4 Relationship between IgA synthesis and plasma progesterone concentration

Intensity of IgA synthesis	+++	++	+	±	0
Nulliparae:-					
Mean plasma progesterone concentration (ng/ml)	-	12.4	7.8	1.3	7.3
± SEM			±2.9		±3.6
Number of specimens	0	1	4	1	5
Parous women:-					
Mean plasma progesterone concentration (ng/ml)	9.2	5.8	7.8	14.9	5.1
± SEM	±2.2	±1.1	±1.7	±2.5	±1.7
Number of specimens	5	9	3	4	3

TABLE 6:5 Relationship between IgA synthesis and plasma oestradiol 17β concentration

Intensity of IgA synthesis	+++	++	+	±	0
Nulliparae:-					
Mean plasma oestradiol 17β concentration (pmol/l)	369	369	568	564	695
± SEM	± 0	± 0	±129	±198	±157
Number of specimens	2	2	7	2	6
Parous women:-					
Mean plasma oestradiol 17β concentration (pmol/l)	661	507	703	923	492
± SEM	±144	±114	±173	±403	±119
Number of specimens	5	9	8	4	7

TABLE 6:6

Influence of diagnosis of primary condition on immunoglobulin synthesis

Histologically normal tissue from patients with localised disease or no disease (Group 1) (38 specimens)

IgA	27 (71%)	5	9	10	3
IgG	16 (42%)	-	2	6	8
IgM	1 (2%)	-	-	-	1

Histologically normal tissue from patients with fibrosing adenosis (Group 2) (42 specimens)

IgA	38 (90%)	4	9	14	11
IgG	20 (48%)	-	-	8	12
IgM	1 (2%)	-	-	-	1

TABLE 6:7 Effect of menstrual age on intensity of IgA synthesis

Menstrual age	Mean intensity of labelling of autoradiographs (number of specimens)				
	+++	++	+	±	0
1 - 9	3	3	2	2	3
10 - 19	3	5	7	7	3
20 - 29	3	6	11	4	6
30 -	0	4	4	1	3
TOTAL: 80 specimens	9	18	24	14	15

TABLE 6:8 Effect of birth interval on intensity of IgA synthesis

Birth interval (yrs)	Mean intensity of labelling of autoradiographs (number of specimens)				
	+++	++	+	±	0
2 - 5	1	0	1	1	0
6 - 10	2	9	3	4	4
11 - 15	4	3	6	4	4
16 - 20	0	2	1	0	1
TOTAL: 50 specimens	7	14	11	9	9

TABLE 6:9 Effect of breast-feeding history on intensity of IgA synthesis

Duration of breast-feeding (weeks)	Mean intensity of labelling of autoradiographs (number of specimens)				
	+++	++	+	±	0
None	3	3	1	5	3
1 - 6	0	5	5	2	1
8 - 20	1	1	2	0	3
24 - 46	1	2	1	0	2
52 - 84	1	3	1	2	0
All breast-feeders	3	11	9	4	6
Total: 48 specimens	6	14	10	9	9

V IMMUNOFLUORESCENT IDENTIFICATION OF IMMUNOGLOBULIN

A) Patients studied

34 specimens were taken from 31 women, all of whom were experiencing regular menstrual cycles. The age range was 19 - 52, with a mean of 33. 15 were nulliparous and 16 had had at least one full-term pregnancy. Three nulliparous subjects and two parous subjects were taking the oral contraceptive pill at the time of the biopsy.

The number of patients studied was limited to 31 because of technical problems or shortage of tissue. The 31 patients studied were part of the larger series of 74 women in whom in vitro synthesis of immunoglobulins was studied. The same exclusions therefore apply - no patient was suffering from breast cancer, and the tissue studied was in each case normal. The same information on reproductive history, date of subsequent period, and plasma progesterone and oestradiol-17β concentrations was available.

B) Histopathology of the primary condition

The diagnosis of the primary condition is shown in Table 6:1(b). In all cases the lesion was benign, and the diagnosis was checked by Dr Smith. Again, the patients were divided into two groups, Group 1 having no disease or localised disease, and Group 2 having fibrosing adenosis, which might not have been localised to the excised lump, even though the tissue examined in this study was normal.

C) Immunological Methods

Tissue blocks were processed by the method of Brandtzaeg (1974). The tissue was washed for 48 hours in cold phosphate-buffered saline to remove tissue fluid proteins, then fixed in cold 96% alcohol for 18 hours and absolute alcohol for four hours, transferred to xylene and embedded

in paraffin. The blocks of embedded tissue were stored at 4°C. Serial sections (6 μm) were stretched on acetone-cleaned glass slides at 40°C by means of a small amount of water which was quickly soaked away. Thereafter the sections were dried at 37°C for 30 minutes and stored at 4°C. Deparaffinisation was carried out the same or the next day at 8-10°C: the slides were finally rinsed briefly in deionised water and air-dried just before incubation.

Tissue sections were incubated with FITC-conjugated rabbit antisera to human α, γ, and μ chains (Hoechst) or with normal rabbit serum as a control for autofluorescence. The specificity of the conjugated antisera was checked by demonstrating a single arc on immunoelectrophoresis against whole human serum and by showing specificity of staining of bone marrow specimens obtained from patients with myeloma of known immunoglobulin class. Control experiments were performed by blocking with unconjugated antisera and by using absorbed conjugated antisera. After 30 minutes' incubation at room temperature, the sections were washed three times in phosphate-buffered saline, mounted in buffered glycerol and examined using a Zeiss fluorescence microscope, with an Osram HB200w lamp, a BG2 excitor filter, barrier filters 50 and 53, and incident illumination. All the sections were examined using a 40x Zeiss objective. Sections were photographed on Kodak Tri-X film with an exposure time of 60-120 seconds.

Sections stained with fluorescent antibody to IgA, IgG and IgM were graded from 0, through + to +++ for: (a) the number of fluorescent plasma cells seen, and (b) the number and intensity of non-cellular deposits. A separate assessment was made of deposits within the lumina of ductules, deposits in the intralobular stroma, and those in the extralobular stroma. Sections in which no lobular tissue could be identified were excluded from the study.

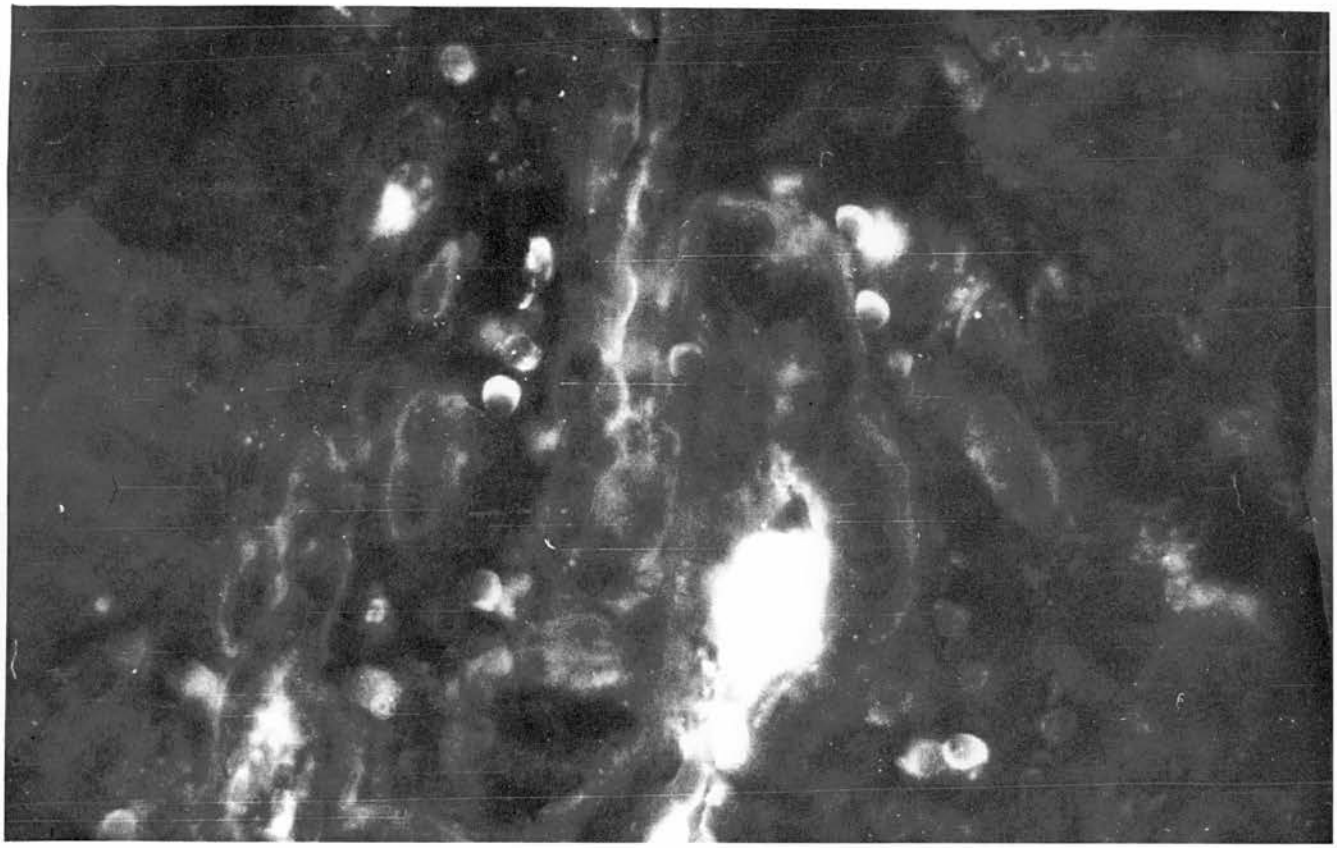


Fig 6:2 Section of breast lobule stained with FITC conjugated anti-IgA showing fluorescing plasma cells. (x 3750).

D) Results

IgA Table 6:10 shows the numbers of specimens which were positive when sections were examined after staining with fluorescent antibody to IgA. Plasma cells were seen in 71% of the sections, and the number of cells was moderate or large in 14 of the 24 positives. In all but two of the sections the cells were seen in greater numbers in the intralobular stroma than in the extra-lobular stroma. Fig 6:2 shows a section graded as +++ for plasma cells.

Deposits of IgA were seen in the lumina of ductules in 88% of the specimens, and as non-cellular foci in the intralobular stroma in 53%. In only five specimens (15%) were deposits seen outside the lobules.

IgG IgG-containing cells were seen in only three sections. Non-cellular deposits of IgG were seen in ten sections (29%), and all were weakly fluorescent or barely visible (+ or ±). More deposits of IgG were seen in the extralobular stroma than in the lobules or the lumina of the ductules.

IgM Fluorescent IgM-containing plasma cells were seen in one specimen, and non-cellular deposits of IgM in three: the IgM deposits showed only barely detectable fluorescence.

Influence of parity and the menstrual cycle Table 6:11 shows the influence of parity and stage of the cycle, and of oral contraceptive use, on two parameters:-

(1) the numbers and intensity of staining of plasma cells in the intralobular stroma (where they were most numerous), and

(2) the amount and intensity of staining of acellular material in the lumina of the ductules (where it was most plentiful). There is a trend

TABLE 6:10

Detection of immunoglobulin in tissue by immunofluorescence

TOTAL: 34 specimens		Number of positive specimens (& % of total)	Mean intensity of labelling of positives (no of cases)			
			+++	++	+	±
IgA						
Plasma cells		24 (71%)	4	10	5	5
Acellular material	(Duct lumen	30 (88%)	6	13	7	4
	(Lobule	18 (53%)	-	2	13	3
	(Stroma	5 (15%)	1	-	2	2
IgG						
Plasma cells		3 (9%)	-	-	2	1
Acellular material	(Duct lumen	4 (12%)	-	-	2	2
	(Lobule	3 (9%)	-	-	3	-
	(Stroma	6 (18%)	-	-	1	5
IgM						
Plasma cells		1 (3%)	-	-	1	-
Acellular material	(Duct lumen	2 (6%)	-	-	-	2
	(Lobule	1 (3%)	-	-	-	1
	(Stroma	1 (3%)	-	-	-	1

towards increased staining of plasma cells in the luteal phase of the cycle, but this is not significant. Oral contraceptives did not have an obvious effect, but the numbers examined were small. There was no correlation with progesterone or oestradiol 17β concentrations (Tables 6:12 and 6:13).

Other factors Tables 6:14 to 6:17 show the influence of diagnosis, menstrual age, birth interval and history of breast-feeding. None of these factors has an obvious effect on the pattern of immunofluorescence, though the numbers of specimens examined are not high. Numbers are too small to allow separate analysis of nulliparae and parous women.

Repeatability of results Immunofluorescence was carried out on three specimens of tissue from the same breast (obtained at reduction mammoplasty). Again the specimens were coded as separate specimens, and none of the observers knew which specimens came from the same patient. IgA-containing plasma cells were seen in two, and graded as + and ±; acellular deposits of IgA in the intralobular stroma were graded as + in all three, and deposits of IgA within the lumina of ductules were + in two and ± in one. Neither cells nor deposits of IgA were seen in the extralobular stroma in any one of the three specimens. No cells or deposits were seen in any of the sections stained for IgG or IgM.

TABLE 6:11

Immunofluorescence: Influence of parity and stage of the cycle

		Intensity of labelling of specimens (number of cases)				
		+++	++	+	±	0
PLASMA CELLS IN LOBULES						
Nulliparae:						
Follicular phase	(8)		2	2	2	2
Luteal phase	(6)		1	2	1	2
Oral contraceptives	(3)		2		1	
Parous women:						
Follicular phase	(6)	1		1		4
Luteal phase	(8)	3	2		1	2
Oral contraceptives	(3)		1	1		1
ACELLULAR MATERIAL IN LUMINA OF DUCTULES						
Nulliparae:						
Follicular phase	(8)	1	2	4	1	
Luteal phase	(6)		3		1	2
Oral contraceptives	(3)	2	1			
Parous women:						
Follicular phase	(6)	3	2		1	
Luteal phase	(8)		5	1	2	
Oral contraceptives	(3)			1	1	1

TABLE 6:12

Relationship between immunofluorescence and plasma progesterone concentration

Intensity of immunofluorescence	+++	++	+	±	0
Plasma cells in lobules:					
Mean plasma progesterone concentration (ng/ml)	8.5	10.4	1.7	13.5	7.7
± SEM	±2.6	±3.7		±4.2	±1.8
Number of specimens	3	3	1	3	4
Acellular material in lumina of ductules:					
Mean plasma progesterone concentration (ng/ml)	-	8.8	7.0	6.0	17.0
± SEM		±1.9		±0.9	±4.2
Number of specimens	0	8	1	3	2

TABLE 6:13

Relationship between immunofluorescence and plasma oestradiol 17β concentration

Intensity of immunofluorescence	+++	++	+	±	0
Plasma cells in lobules:					
Mean plasma oestradiol 17β concentration	753	356	741	472	704
± SEM	±368	±130	±127	±98	±181
Number of specimens	3	5	7	4	9
Acellular material in lumina of ductules:					
Mean plasma oestradiol 17β concentration (pmol/1)	707	562	642	378	781
± SEM	±274	± 94	±180	±8	±214
Number of specimens	6	10	7	2	3

TABLE 6:14 Relationship between diagnosis of primary condition and immunofluorescence

	Intensity of immunofluorescence (numbers of specimens)				
	+++	++	+	±	0
Plasma cells in lobules:					
Group 1 (16 specimens)	1	2	4	2	7
Group 2 (18 specimens)	3	6	2	3	4
Acellular material in lumina of ductules:					
Group 1 (16 specimens)	4	5	3	3	1
Group 2 (18 specimens)	2	8	4	2	2

TABLE 6:15 Relationship between menstrual age and immunofluorescence

	Intensity of immunofluorescence (numbers of specimens)				
Menstrual age	+++	++	+	±	0
Plasma cells in lobules:					
1 - 9	0	0	2	2	1
10 - 19	1	2	1	3	4
20 - 29	3	5	1		4
30+	0	1	2	0	2
Acellular material in lumina of ductules:					
1 - 9	0	1	2	1	1
10 - 19	1	5	1	2	2
20 - 29	3	5	4	1	0
30+	2	2	0	1	0

TABLE 6:16

Relationship between birth interval and immunofluorescence

	Intensity of immunofluorescence (numbers of specimens)				
Birth interval (years)	+++	++	+	±	0
Plasma cells in lobules:					
2 - 5 (1 specimen)					
6 - 10 (8 specimens)	2	1		1	4
11 - 15 (6 specimens)	2		2		2
16 - 20 (2 specimens)		2			
Acellular material in lumina of ductules					
2 - 5 (1 specimen)		1			
6 - 10 (8 specimens)		3	2	3	
11 - 15 (6 specimens)	3	1	1		1
16 - 20 (2 specimens)		2			

TABLE 6:17

Relationship between breast-feeding and immunofluorescence

	Intensity of immunofluorescence (numbers of specimens)				
Breast-feeding history	+++	++	+	±	0
Plasma cells in lobules					
Did not breast-feed (7)	2	1	1		3
Breast-fed (9)	1	2	1	1	4
Acellular material in lumina of ductules					
Did not breast-feed (7)	0	2	2	2	1
Breast-fed (9)					

DISCUSSION

IgA is the principal immunoglobulin in most human external secretions, including colostrum and milk. By comparison, the principal circulating immunoglobulin is IgG. It has been known for many years that the IgA in salivary and intestinal secretions is synthesised locally by subepithelial plasma cells (Tomasi and Bienenstock, 1968). During lactation, many plasma cells are seen in the human breast (Beer et al, 1974), and it has therefore been assumed that the IgA in human milk is also locally synthesised. Local synthesis of immunoglobulin has been shown in several animal species (Lascelles and McDowell, 1974; Watson and Lascelles, 1973), but as mentioned above, little is known about immunoglobulin synthesis in the human breast (Hochwald et al, 1964).

It is clear from the results presented above that the non-lactating breast produces IgA rather than other immunoglobulins. This suggests that the synthesis of IgA is not a non-specific inflammatory response but a process associated with secretion. The interpretation that it is a low-grade secretory process is strengthened by the finding that plasma cells containing IgA are localised to the lobules rather than being randomly spread through the gland. Deposits of IgA are also concentrated in the lobules and particularly in the lumina of the ductules, again suggesting a process of active secretion of the immunoglobulin into the ductules. In comparison, the cells containing IgG are fewer and are randomly distributed throughout the lobules and the stroma.

Since many of the specimens of breast tissue were obtained from patients with breast nodules, it is possible that the secretion of IgA is a reaction to breast disease. However, similar results were obtained with mammoplasty specimens, and so it appears likely that IgA synthesis is a property of normal breast tissue.

It may be that the secretion of IgA into the ductules is a form of defence against infection (mastitis being very rare in the non-lactating breast), but it is also possible that it represents a low level of activity in a gland whose primary function is full lactation. Amongst primitive human beings the breast was probably lactating almost continuously, with interruptions only for pregnancy, and it is therefore possible that humans have not evolved a mechanism to "switch off" secretion completely.

Synthesis of IgA occurred in both parous and nulliparous women, but cyclical changes in the amount of synthesis were found only in parous women. The amount of IgA synthesis increased markedly in the luteal phase of the cycle, implying that this is due to an effect of progesterone. The observation that the increase in IgA synthesis was not seen in nulliparae would suggest that before first pregnancy the breast is less sensitive to progesterone. This agrees with the findings in Chapter 5, and will be discussed further in the final chapter.

The increased amount of IgA synthesis may be due to either increased numbers of plasma cells or increased activity of the plasma cells already present. The examination of MGP-stained secretions indicated that there are increased numbers of plasma cells, but it is difficult to tell whether or not there is also increased activity. The mechanism for attracting circulating plasma cells into the mammary gland is still poorly understood, although this is being investigated. Mattioli and Tomasi (1973) labelled plasma cells by injecting tritiated thymidine into neonatal mice, and checked the disappearance of labelled cells: they estimated the half life of IgA plasma cells from the gut

at 4.7 days, and the maximum life span as eight weeks. Weisz-Carrington et al (1978) found that in mice, a combined regimen of progesterone, oestrogen and prolactin produces development of glandular epithelium with increased numbers of IgA-secreting plasma cells and increased intra-epithelial IgA. This appeared to be due to an increased ability of the gland to attract and/or retain precursors of IgA plasma cells derived from gut-associated lymphoid tissue.

It is possible that the stimulus for increased IgA synthesis is the increased secretion of a local transmitter from the breast epithelial cells, but until such a transmitter can be identified this possibility will remain unproved. However, since there is no other evidence to suggest that plasma cells themselves are sensitive to ovarian steroids, it seems more likely that their increased number in the breast at certain times in the cycle is due to increased secretion of a transmitter by the epithelium - ie. that immunoglobulin synthesis is an indirect indicator of epithelial cell activity.

SUMMARY

80 specimens of macroscopically and histologically normal breast tissue were examined by in vitro culture for immunoglobulin synthesis. 34 specimens were examined by immunofluorescence for the presence of immunoglobulins. Synthesis of IgA was detected in 81% of specimens, while synthesis of other immunoglobulins was much less intense. Plasma cells containing IgA were seen in 71% of the specimens examined by immunofluorescence, and 88% of specimens had deposits of IgA in the ductules. Nulliparous women showed no cyclical changes, but among parous women IgA synthesis was more intense during the luteal phase of the cycle. Other factors did not affect the intensity of immunoglobulin synthesis.

Chapter 7

THE MENSTRUAL CYCLE AND PARITY IN THE AETIOLOGY OF BREAST CANCER

The results of the four parts of this study - morphological, histological, DNA-labelling and immunological - have been discussed in the appropriate chapters. The purpose of this final chapter is to compare these results with one another, and to discuss their relevance to theories about the hormonal control of breast cancer.

This chapter consists of three sections. First, the results of the research are summarised and compared. Second, the epidemiology of breast cancer is reviewed and current theories about its hormonal aetiology are discussed. Finally, a new hypothesis is put forward based on the results of this work.

1 THE EFFECT OF PARITY AND THE MENSTRUAL CYCLE ON THE BREAST

A Summary of results

In the histological investigation described in Chapter 4, the most striking positive finding was the difference between parous and nulliparous women. This difference had not been investigated before. The difference between the two groups was just significant as far as lobule density was concerned, but highly significant as far as types of epithelium were concerned. The finding of a difference in epithelial types agrees with the broad conclusions of Chapters 5 and 6 - that there is a difference between parous and nulliparous women - but gives no indication of what the functional difference might be, since the function of the clear cells (which are commoner among nulliparae) is unknown.

Cyclical changes with the menstrual cycle (first suggested by Rosenburg in 1922) were limited to changes in epithelial height, and these changes were seen among both parous and nulliparae. Budding of

the ductules does not occur. The change in epithelial height draws attention to the action of ovarian steroids on the epithelium itself, but the observation that changes occur among both parous women and nulliparae is in contrast to the cyclical changes noted in Chapters 5 and 6, which occurred among parous women but not among nulliparae. Again, the histological picture does not indicate what functional changes may be occurring, other than a probably generalised increase in cell activity during the luteal phase of the cycle.

The suggestion by Dieckmann (1925) that the breast changes with age is not confirmed by the histological study: the mammary gland is less fully developed soon after the menarche than it is in later years, but it does not continue to develop all through the reproductive years. On the contrary, the diameter of the ductules appears to become less as the woman becomes older.

Despite the observation that histological changes during the cycle are limited to the epithelium only, the morphological study described in Chapter 3 demonstrates that cyclical changes in breast volume occur in nulliparae. It seems likely that similar changes occur also among parous women, although this has not been investigated. The amount of the change is of the order of 20%, and it is therefore not due to changes in the breast lobules - which frequently constitute well under 20% of the volume of the breast. The volume change is probably caused by alteration in the fluid content of the stroma, and this fluid may be intravascular or extravascular, or both. Although no changes in stromal oedema were detected in the histological examination, a 20% change might well be undetectable histologically.

The studies of DNA synthesis (Chapter 5) and immunoglobulin synthesis (Chapter 6) agree in their conclusions: functional changes - unlike structural changes - occur during the cycle among parous women, but not among nulliparae. Both these parameters are probably indicators of epithelial cell activity. DNA synthesis is a direct indicator, implying preparation for cell division - in preparation for either ductule proliferation or increased cell turnover. (In this context it is interesting to note that the biopsy taken during the seventh week of pregnancy showed no proliferation of the ductules). Immunoglobulin synthesis may be an indirect indicator of epithelial cell activity: although due directly to an increase in the number of plasma cells, this increased number of plasma cells probably reflects an increased secretion of a local attractant - perhaps by the epithelial cells of the ductules. Thus the cyclical changes in immunoglobulin synthesis may indirectly indicate cyclical changes in the production of the transmitter by the epithelium.

In both Chapter 5 and Chapter 6 the numbers of nulliparous women examined were small, and so there exists the possibility that cyclical changes were not observed among nulliparae because too few specimens were examined. It is difficult to know how many specimens would have to be examined before it could be said with certainty that no cyclical changes existed. Nevertheless, the fact that both chapters agree in showing cyclical changes among parous women and none among nulliparae supports the idea that the observations are genuine.

In each of the four parts of the study, the increases that were observed during the cycle occurred during the luteal phase. Thus they appear to be a response to increased plasma progesterone

concentration. No other hormone, ovarian or pituitary, increases at this stage of the cycle to a higher level than at any other stage (apart, of course, from progesterone derivatives). Oestrogen levels are highest during the follicular phase of the cycle, when breast volume, epithelial height, DNA synthesis and IgA synthesis were lowest. The observation that DNA synthesis and IgA synthesis do not show cyclical changes among nulliparae therefore indicates that the epithelium of the ductules among nulliparous women does not respond to progesterone as completely as that of parous women. The level of epithelial cell activity among nulliparae (as indicated by DNA and IgA synthesis) is not uniformly low, and so the cells are being stimulated to some extent throughout the cycle - but this stimulation is not regularised as it is among parous women.

B Conclusions

The conclusions of the various studies may be summarised as follows:

1 Histologically, there is a difference between the ductular epithelium of nulliparous women and that of parous women.

2 Among nulliparous women, the stroma of the breast and the ductule epithelium appear to be hormone sensitive.

3 Among nulliparous and parous women, a low level of epithelial cell activity is present throughout the cycle (as shown by DNA and IgA synthesis).

4 Among parous women, epithelial cell activity varies during the menstrual cycle, apparently in response to fluctuations in the plasma progesterone concentration.

5 Among nulliparous women, no cyclical change in epithelial cell activity occurs in response to fluctuations in plasma progesterone concentration.

6 Therefore, although the epithelium of the nulliparous woman is active and hormone sensitive, it is less sensitive to progesterone than is the epithelium of the breast of a parous woman.

A change in the hormone sensitivity of the breast as a result of parity may have a bearing on the breast's susceptibility to malignant change. Before this is discussed, the epidemiological data relating to hormones and breast cancer will be reviewed.

II EPIDEMIOLOGY OF BREAST CANCER

A General Factors

The human is the only primate in which breast cancer is common (Seibold and Wolf 1973), but the reason for this is unknown. In the human, breast cancer is the commonest malignancy affecting the female, and its incidence is increasing: in the USA it is now 82.7 per 100,000 woman-years. The results of treatment have changed little since 1935 (Henderson and Canellos 1980), and the mortality rate has scarcely improved over the last 40 years - a slight decrease (24.9 to 23.3 per 100,000 woman-years) has been attributed to earlier diagnosis (Brian et al 1980). During the last 20 years the epidemiology of the condition has been extensively studied.

1 Geographical factors

Breast cancer is commonest in North America and Western Europe (Levin and Thomas 1977), and its incidence declines in Eastern Europe and Asia to a low level in Japan (Doll 1972). Migration from an area of low incidence to one of high incidence (eg. from Japan to California) appears to increase an individual's risk of cancer (Buell 1973). The difference in incidence are therefore not genetic in origin. The reason for them in unknown. It has been suggested that ingestion of

dietary fat is a possible environmental factor (Miller 1978; Wynder 1980). In experimental animals high-fat diets promote both the appearance and the growth of mammary tumours: this effect may be mediated by prolactin. Breast cancer incidence can be correlated with fat intake on a national level but a cause-and-effect relationship is far from proved.

De Waard et al (1977) suggest that part at least of the difference in incidence between Japan and Holland can be explained by differences in body weight and height. They found that among Dutch patients with breast cancer, those with metastases were heavier than those without nodal involvement. Beer and Billingham (1978) suggested that the difference in breast cancer incidence between Caucasian and Oriental women may be due simply to breast size differences: the larger amounts of adipose tissue in the breasts of Caucasian women might act as reservoirs and slow-release depots for blood-borne carcinogens. However, breast size does not differ between breast-cancer patients and controls (Adami and Rimsten 1978); Petrakis and Ernster 1978). Petrakis and Ernster point out that blood-borne carcinogens would be stored and released as effectively by distant fat stores as by the breasts themselves.

Trichopoulos et al (1980) point out that there are differences in breast cancer incidence within populations also: higher social class women have 2-4 times the risk of developing the disease compared to lower socio-economic groups.

The effects of age on breast cancer incidence have been summarised by Doll (1972). The condition is extremely rare under the age of 25. In areas of low incidence the maximum incidence is at age 55, but

in areas of high incidence such as Great Britain, the incidence of the disease increases steadily with age, though the rate of increase is slower after the menopause. The significance of the menopause is emphasised by a slight decline in incidence between ages 45 and 54, before the rising pattern is again resumed. There has been a slight increase in the incidence of breast cancer in this century, particularly during the last ten years; this is particularly seen in the 55-64 age group (and is therefore not the result of oral contraception). Brian et al (1980) found a 25% increase in the age-adjusted incidence rate in Rochester, Minnesota, between 1935 and 1974, most of this increase occurring in women aged between 45 and 64.

2 Breast-feeding

The influence of breast-feeding on subsequent cancer incidence was first studied in a controlled way by Lane-Claypon in 1926, though the first recorded suggestion of a connection was 80 years earlier (Walshe 1846). Lane-Claypon reported that a history of breast-feeding was commoner among controls than among cancer patients. More recent studies have failed to confirm this finding (MacMahon and Feinlieb 1960), though Levin et al (1964) felt that there was some effect of lactation - with short histories of lactation (up to 17 months) increasing the risk slightly, and longer histories causing a decrease. Lilienfeld (1963) noted the conflicting results of different investigations of this question, and made the point that "if nursing habits played an important etiologic role in breast cancer, one would expect marked changes in the incidence" of the disease as a result of different lactation patterns. He also suggested that the effect of lactation might be explained not by a positive influence of lactation itself but rather by the fact that lactation suppresses menstruation, and the menstrual cycle may have the more important effect.

The strongest evidence in favour of the possibility that breast-feeding in itself affects the incidence of subsequent breast cancer is seen in a report from Hong Kong (Ing et al 1977). Women in fishing villages there breast-feed only from the right breast, and among 73 such women who developed breast cancer, the tumour was left-sided in 27 of 35 (79.4%) post-menopausal women (ie. women over 55 years old), and left-sided in 48.7% of the 39 pre-menopausal women. However, McManus (1977) pointed out that many other large studies of the laterality of breast cancer have also found a predominance of left-sided tumours. He also pointed out that the gene pool of the Tanka women may be significant - it is known from large surveys that a relative is more likely to be affected in the same breast only if the presenting case has a left-sided tumour. Finally, he points out that adeno-carcinoma is for some reason much commoner on the left. A recent report from Oxford (Kalache et al 1980) found no relationship between breast-feeding history and breast cancer. The report compared 707 patients under 50 years of age with 707 matched controls: no differences were found in the numbers of women breast-feeding or the duration of lactation.

3 Pregnancy and menstruation

The importance of parity has long been recognised: a nulliparous woman has a greater risk than a parous woman of developing breast cancer. Janerich (1978), reviewing New York State Cancer Registry data, found that this relationship holds only for older women, and that breast cancer in younger women is commoner among married women. To explain this, he pointed out that malignant cells are similar to fetal cells, and suggested that immunosuppression during pregnancy allows tumours to grow and that many of these tumours are then destroyed when the immune system returns to normal after pregnancy:

if the tumour has become too large, however, its destruction will be impossible. Doll (1978) pointed out that the figures on which this theory is based are probably a statistical artefact. However, the possibility remains that altered immune tolerance during pregnancy may have some bearing on its effect on breast cancer incidence.

In a world-wide survey comparing breast cancer patients with controls, another factor emerged - the age at which a woman has her first full-term pregnancy (MacMahon et al 1970). If this occurs before a woman is 20 years old, her risk of subsequent breast cancer is less than half that of a nulliparous woman. There is a linear relationship between the age at first birth and the risk of breast cancer, between the ages of 20 and 33, after which the protective effect of parity is no longer seen. After the age of 33 a first pregnancy appears actually to increase the risk of subsequent breast cancer (see Fig 7:1). The suggestion that late first pregnancy increases the risk of subsequent breast cancer was cautiously interpreted when the data were first published (MacMahon et al 1970), and the authors emphasised that the points lying above the line, denoting increased risk, necessarily represented small numbers of patients, as few women have their first pregnancy late in reproductive life. However, the consistency of the results is striking, and in a later review the authors themselves appeared more convinced that these data points are significant (MacMahon et al 1973).

A number of studies, such as that of Levin et al (1964), have confirmed the suggestion of Lilienfeld (1963) that the total number of menstrual cycles is related to the risk of breast cancer. Late menarche and early menopause both have significant protective effects on the risk

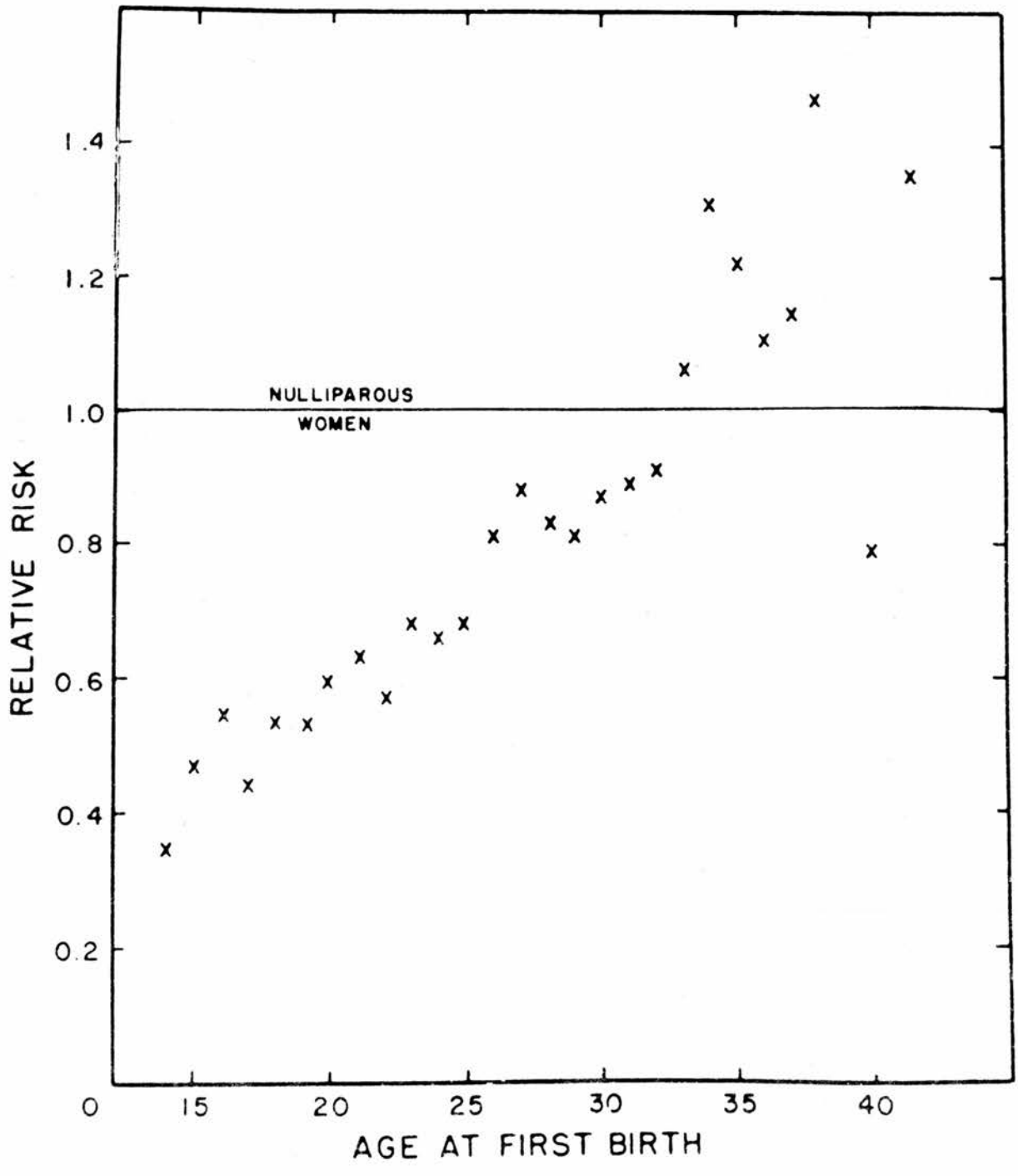


Fig 7:1 Relative risk of developing breast cancer in relation to the mother's age at the birth of her first child. The risk in nulliparous women is taken as 1.0. (From MacMahon et al 1970).

of subsequent breast cancer (Doll 1975), the effect of the latter being much greater than that of the former. In fact, an artificial menopause under the age of 35 reduces the risk to about one-third of that in women having a natural menopause between the ages of 45 and 55, and a delayed natural menopause after the age of 55 increases the risk by one-half (Trichopoulos et al 1972; Doll and Smith 1968).

The significance of the age at first birth was confirmed by a large scale study of 20,000 women in New York (Shapiro et al 1973), and this study also confirmed the lack of significance of total family size. It confirmed too the increased risk associated with early menarche - menarche at ages under 15 was found to be associated with a 50% increase in relative risk - but did not confirm the importance of menopausal age: early menopause seemed to have a protective effect, as reported by others, but the numbers in this series were small and the effect was not significant.

Farewell (1977), studying the incidence of breast cancer in Guernsey, concluded that age at menarche, age at first birth, family history and aetiocholanolone secretion are important risk factors and have additive effects on breast cancer risk. However, Adami et al (1978), in a case-control study of 179 consecutive breast cancer patients in Sweden, were unable to confirm any relationship between breast cancer risk and age at menarche, age at first birth, age at menopause or number of children - either in pre- or post-menopausal women.

Miller (1978) was also unable to confirm the significance of age at first pregnancy or early menarche in a case-control study in four areas of Canada, but Ravnihar et al (1979) confirmed the significance of age at first birth in a case-control study in Yugoslavia. A case-control study of 236 breast cancer patients in Utah (Hunt et al 1980)

found that a late age at first birth was strongly associated with breast cancer incidence, and also that risk was correlated with the age at last delivery.

The effect of age at first birth appears to explain some of the previous confusion about the influence of parity and lactation. Women who have their first pregnancy early in life are more likely to have larger families and therefore are also more likely to breast-feed for a larger part of their lives. When the age at first birth is taken into account, however, lactation had no effect on breast cancer risk - neither the absence or presence of lactation, nor its average duration, nor its prolongation for more than 24 months (MacMahon et al 1970). However, the effect of age at first birth does not fully explain the effect of socio-economic status - social classes I and II have some 50% greater risk of breast cancer than social classes IV and V. Nor does it explain the variation in breast cancer incidence in different countries (MacMahon and Cole 1972) - though the effect of the age at first birth is seen in all areas of the world, whether of high or low breast cancer incidence.

4 Other factors

The New York study (Shapiro et al 1973) also confirmed the importance of a family history of breast cancer - there was a 30% increase in risk if a mother or sister had the disease. An extensive study of twins in the Danish Twin Registry did not produce any firm conclusion on the heritability of breast cancer (Holm et al 1980). These authors estimated the Genetic Determination of breast cancer is approximately 0.30 - 0.40, but emphasised that this estimate was liable to error, and concluded that "genetic factors seem to be of some importance in the development of breast cancer, whereas these factors play no

major role in the development of cancer of other sites".

Shapiro et al (1973) showed an association between benign breast conditions and breast cancer - a prior "benign breast lesion" was associated with a 60-100% increased risk of breast cancer. Previous investigators had found an association between breast disease and subsequent breast cancer (Warren 1940; Lewison and Lyons 1953). The estimates of the increased risk vary between a doubling of risk (Davis et al 1964) and a five-fold increase (Black et al 1972), but the latter figure is based on a search for "atypical characteristics" within biopsies of benign lesions. The term "benign breast disease" covers a large number of histological conditions (Davis et al 1964) and there have been a number of attempts to define the apparently benign changes which are associated with a higher risk of subsequent breast cancer (Foote and Stewart 1945; Karpas et al 1965; Wellings 1980). These attempts have been hampered by a lack of consensus regarding terminology: each survey has had to define its own criteria for diagnosis of each lesion, and this difficulty not only complicates comparison of different studies, but also reflects the range of opinion among pathologists when asked to classify any particular specimen. However, in a 13-year follow-up of 1441 patients with benign breast disease, Hutchinson et al (1980) found 66 cases of breast cancer - an incidence of 3.55 per 1000 woman-years (which is 2.1 times that in the general population). They found that fibroadenoma alone was not associated with increased risk, while fibrocystic disease was: in women with fibrocystic disease increased risk was related to the presence of epithelial hyperplasia or papillomatosis, especially if there was calcification. Larger and bilateral lesions carried increased risk.

The relationship between low-dose radiation and breast cancer has been intensively studied (Land 1980) but this is thought not to be a very important factor in the aetiology of the disease. However, the observation that radiation may be especially dangerous at adolescence (as shown by the incidence of breast cancer among survivors of the atomic bombing in Japan) strengthens the idea that breast cancer initiation occurs at this time (Cole 1980).

The possibility of a viral aetiology remains open, though it is now thought unlikely (Cole 1980). However, the identification in human breast cancer of an antigen similar to mouse mammary tumour virus has recently been reported (Spiegelman et al 1980).

B Endocrine factors

The observations that both the age at menarche (Stascewski 1971) and the age at the menopause have an effect on breast cancer risk point to a connection between endogenous hormone levels and breast cancer. The total number of years of menstrual activity is related to breast cancer risk (Levin et al 1964), with a greater risk being associated with more than 35 years of menstrual activity than with less than 30 years. It is now generally agreed that ovarian hormones are implicated in the initiation or promotion of breast cancer, and most interest so far has centred on the role of oestrogens.

1 Oestrogens

MacMahon and Cole (1972) summarised the evidence for ovarian involvement in breast cancer. They concluded that "the proportion of human breast cancer cases that depend on an intact ovary for their development is at least two-thirds and may be even higher", basing this conclusion on the effect of early surgical menopause in reducing risk of

subsequent breast cancer by two-thirds, and on the fact that in dogs neutering reduces the risk of breast cancer by about 90%. Addressing the question of whether oestrogens affect tumour induction or promotion, they point out that the importance of the age at menarche suggests that ovarian activity has a role in tumour induction. The years immediately after the menarche may be years of high risk of tumour induction, and the significance of the age at first birth suggests that this high-risk period terminates with first pregnancy. They found little evidence that the ovary also has a role in tumour enhancement.

MacMahon and Cole developed a theory that the carcinogenic effect of ovarian activity depends on the type of oestrogens produced by the ovary: oestriol has not been demonstrated to have the same carcinogenic effect as oestrone or oestradiol, and MacMahon and Cole suggest it has a protective effect against the other two oestrogens. Lemon (1970) postulated that oestriol competes for oestradiol receptors in breast tissue and thus impedes the oestradiol stimulus to breast cancer induction. Such a protective role has been demonstrated in the rat (Terenius 1971). In support of their hypothesis, MacMahon and Cole have shown, in a study of urinary "oestriol ratios" among different groups of women, that the ratio of oestriol to the other two oestrogens is much higher in young Asian women (who have a low risk of breast cancer) than it is in young American women. Trichopoulos et al (1980), in a recent study in Athens, correlated urinary oestriol ratios with social class: the lower socio-economic group (with less risk of developing breast cancer) had 50% higher oestriol ratios than the higher socio-economic group.

The "oestriol hypothesis" has been criticised on various grounds. Smith and Smith (1970) suggested that the carcinogenic potential of oestrogens may be indirect, acting through the medium of pituitary hormones, but they offered little evidence in support of this idea. Kirschner (1977) reviewed some of the criticisms: a number of investigators have found increased urinary oestriol excretion among breast cancer patients; circulating oestriol levels are normally too low to modulate the action of oestradiol or oestrone; and perhaps most importantly the excretion of oestriol does not accurately reflect its production. Longcope and Pratt (1978) compared groups of women with different urinary oestrogen ratios and found no differences in the ratios of blood production rates of oestriol, oestrone and oestradiol. Kirschner (1977) also points out that the hormone profiles in older women may not reflect the hormone profile at a younger age, when the cancer was initiated.

Siiteri et al (1974) presented an alternative "oestrone hypothesis": they suggest that oestrone is the important oestrogen in carcinogenesis since it is the oestrogen exclusively produced by postmenopausal and anovulatory women "having constitutional stigmata commonly encountered in endometrial cancer patients", and is concentrated by breast carcinoma after superfusion. They point out that oestriol competes more effectively for receptors with oestrone than with oestradiol, and add that their hypothesis can be tested by observing the effects of exogenous oestrogens on breast cancer incidence: if oestrone is indeed the main carcinogen then the administration of oestradiol and progesterone will decrease the incidence of cancer.

Such a decrease has not been observed. Hoover et al (1976) found that prolonged administration of exogenous oestrogens appeared to increase the risk of breast cancer, though the effect was not dose-related. Thus breast cancer appeared to respond to exogenous oestrogen administration in the same way as endometrial cancer does (Smith et al 1975); Ziel and Finkle 1975), and although the risk of breast cancer is apparently not great (and only appears after at least ten years' exposure to oestrogens), the fact that no decrease in incidence has occurred appears to weaken the "oestrone hypothesis".

Cohen et al (1978) have suggested that the hyperoestrogenism associated with breast cancer may be caused by diminished production of melatonin by the pineal gland. Pineal calcification is apparently commonest in areas with high breast cancer incidence: melatonin can act on the ovary and its impaired secretion is believed to trigger puberty. Melatonin may influence tumour induction in experimental animals (Karmali et al 1978). The significance of pineal calcification has been questioned, however, by Tapp (1978), who suggests that hyper-function of the pineal is associated with breast cancer, through increased prolactin secretion. Evidence for both these hypotheses is slender.

Nandi (1978) suggested that the role of oestrogens in carcinogenesis is synergistic with other carcinogens and is a dual role: first, the hormones are necessary for the growth of cells transformed by other carcinogens and second, by increasing the rate of cell division and shortening the life-span of normal cells, they reduce the ratio of normal to transformed cells.

There now appears to be some disenchantment with the numerous theories proposed on the roles of oestrogens in carcinogenesis. Sherman et al (1979) found that oestradiol levels during the cycle were normal in a group of breast cancer patients. Korenman (1980) noted that endocrine studies have produced inconsistent results and invite scepticism, and he made the same suggestion as Nandi (1978), that the endocrine environment influences the breast's susceptibility to other carcinogens. More interest is now being shown in the role that progesterone may play in mammary carcinogenesis.

2 Progesterone

Grattarola (1964), in a study of the premenstrual endometrial patterns of women with breast cancer, found that they had an increased incidence of anovulatory cycles. He looked at 87 premenopausal women with breast cancer, and performed endometrial curettage 24-48 hours before the menstrual period was due: he compared the results with the examination of 59 women with normal breasts, having a similar age range. Cycles were ovulatory in 67.7% of the normal women but only 17.2% of the patients with breast cancer. It is of course possible that since the specimens were obtained just before or just after mastectomy the psychological stress of the discovery of cancer had interfered with normal ovulation.

Later evidence on the role of progesterone is not all in agreement. Trichopoulos et al (1980) found that anovular cycles were indeed less frequent in a lower socio-economic group of women, at less risk of breast cancer, but Sherman (1979) found that progesterone production during the cycle was normal in a group of breast cancer patients. Wallace et al (1978), in a survey of menstrual cycle patterns, found that late menarche (which should protect against breast cancer) was

associated with longer and more variable cycles - though these workers did not confirm that these variable cycles were anovulatory. Age at menarche, they found, did not affect fertility, and they also noted that a long period of irregular cycles usually precedes a late menopause (which increases the risk of breast cancer).

Sherman and Korenman (1974) suggested that increased frequency of anovulatory cycles may explain the connection between some well-recognised risk factors and breast cancer. As well as noting that menstrual cycles after the menarche and before the menopause are anovulatory (which may explain the importance of early menarche and late menopause in increasing breast cancer risk), they point out that nulliparity (another risk factor) may be due to anovulation; that obesity (also a risk factor) is associated with anovulation; and that inadequate corpus luteum function would result in diminished urinary androgen excretion - another factor associated with increased risk of breast cancer (Bulbrook et al 1971: see below). Inadequate production of progesterone would mean that the breast was subjected to "unopposed" stimulation by oestrogens. Although the nature of the modification of the action of oestrogen by progesterone is not understood, it is now being recognised as far as the endometrium is concerned that unopposed oestrogen stimulation may be carcinogenic - and that this carcinogenicity may be modified by intermittent exposure of the endometrium to progesterone (Sturdee et al 1978; Craft et al 1978; Paterson et al 1980).

Sherman and Korenman's hypothesis has received support from the Guernsey study (Bulbrook et al 1978); premenopausal women at high risk of breast cancer have lower plasma progesterone levels during the luteal phase of the cycle than normal women, although they have normal oestradiol levels.

Korenman has recently developed his "inadequate luteal phase" theory into a so-called "oestrogen-window" hypothesis (Korenman 1980a,b). He suggests that unopposed oestrogen stimulation at the time of the menarche and the menopause opens a window to other carcinogens - a window that is effectively closed at other times by the presence of progesterone. His hypothesis has five parts:

1) Human breast cancer is induced by environmental carcinogens in a susceptible breast.

2) Unopposed oestrogen stimulation is the most favourable state for tumour induction.

3) There is a long latent period between tumour induction and clinical expression.

4) The duration of the exposure to oestrogens determines risks.

5) Susceptibility to induction ("inducibility") declines with the establishment of normal luteal phase progesterone secretion and becomes very low during pregnancy.

In support of his hypothesis, Korenman cites the incidence of breast cancer among atomic-bomb survivors in Japan: those irradiated at the age of 10-14 showed the highest incidence of subsequent breast cancer, but those aged between 30 and 49 at the time of exposure showed no subsequent effect. According to this hypothesis, the administration of exogenous post-menopausal oestrogens would extend the second window.

3 Oral Contraceptives

As yet no effect of oral contraceptives on breast cancer incidence has been shown by epidemiological studies (Arthes et al 1971; Royal College of General Practitioners 1974; Sartwell et al 1977; Vessey 1978). Lesnick (1971) reported that the incidence of breast cancer in women taking the Pill was less than expected. No morphological

differences have been seen in cancers in women taking the oral contraceptive pill (Fechner 1970c) or oestrogen replacement therapy (Fechner 1972b). Fechner (1972a) also found no difference between benign breast disease in women taking post-menopausal oestrogen replacement therapy. Thomas (1978), summarising nine case-control studies or oral contraceptives and breast cancer, comments that their consistency in showing no effect is striking, considering the numerous methodological differences between the studies.

However, it has been suggested that the Pill may have some effect in increasing cancer risk in nulliparous women only (Black and Leis 1972), and that oral contraceptives greatly increase the risk of breast cancer in women who have had previous benign breast disease (Fasal and Paffenbarger 1975). Paffenbarger (1977) suggested that the use of oral contraceptives before first pregnancy may increase the risk of breast cancer; an effect which may be due to their delaying the first pregnancy rather than to their effect on the breast.

Recently, however, a Californian case-control study (Pike et al 1981) of 163 young women aged 32 or less at the time of diagnosis of breast cancer showed a relationship between cancer risk and the number of months of taking oral contraceptives before first full-term pregnancy. The risk was increased 2.2 times at six years of use. By contrast, oral contraceptive use after first full-term pregnancy was not associated with any increased risk. The same study suggested that a first-trimester abortion before first full-term pregnancy was also associated with increased risk (2.4 fold). This study has been criticised (Royal College of General Practitioners 1981) because only 163 of an eligible 245 patients were interviewed, and because it is not quite clear

whether the study controlled for the interval between menarche and first pregnancy.

Nevertheless, a similar trend is shown by another study (Royal College of General Practitioners 1981). Although this survey (which covers 23,000 women using the Pill and 23,000 controls) found no overall relationship between Pill-use and breast cancer, the risk ratio in women under 35 years old at diagnosis was 2.81. This ratio was not significant, but in the age-group 30-34, the ratio did reach significance, at 3.33. The Oxford-Family Planning Association study showed no such trend (Vessey et al 1981), but the latter authors point out that the Oxford study is based mainly on women who were older and who tended already to have had a full-term pregnancy when they started taking oral contraceptives. The significance of these studies is still uncertain, but may become clearer with a longer period of observation.

There seems to be agreement on the observation that oral contraceptives decrease the risk of benign breast disease (Fasal and Paffenbarger 1975; Vessey et al 1972). The connection between benign breast disease and cancer (mentioned above) is not a strong one, and it seems that the decrease in incidence of benign breast lesions is not mirrored by a decrease in incidence of carcinoma. There have been isolated reports of unusual fibroadenomas removed from women on the Pill (Brown 1970; Goldenberg et al 1967), but no differences were found in controlled series between lesions from women on oral contraceptives and controls (Taylor 1971; Fechner 1970a,b). Erb and Kallenberger (1972) found that oral high-dosed oestrogen-progesterone combinations tended to produce adenosis, but there was less cystic disease and no sign of precancerous change.

In a case-control study in Yugoslavia, Ravnihar et al (1979) confirmed the finding that oral-contraceptive use is associated with a decreased incidence of benign breast disease but does not affect the incidence of breast cancer. LiVolsi et al (1978), in a histopathological study of 205 premenopausal women, attempted to resolve the apparent conundrum that the Pill lowers the incidence of benign breast disease while not affecting the incidence of cancer, despite the fact that there is normally a correlation between benign breast disease and cancer (Hutchinson et al 1980). LiVolsi's conclusion was that oral contraceptives protect only against fibrocystic disease in which epithelial atypia was minimal or absent, and they do not protect against premalignant forms of benign breast disease.

4 Androgens

The possibility that androgens have a role in the initiation or promotion of breast cancer was raised by the work of Bulbrook et al (1971). These investigators set up a prospective study in the island of Guernsey, measuring urinary androgen and corticosteroid metabolites in 5000 healthy women. When breast cancer was later diagnosed in a woman taking part in the study, her urinary excretion of these steroids was retrospectively compared with that of matched controls. Bulbrook's group found that the 27 breast cancer patients had much lower levels of androsterone and aetiocholanolone in the urine than did 187 matched controls. They do not offer an hypothesis to explain this, but Kirschner (1977) has suggested two possibilities: one (that the decreased excretion is the result of nutritional, thyroidal or other factors) seems unlikely to be correct several years before the appearance of the disease; and the other is that androstenedione, which is normally excreted as aetiocholanolone,

is almost completely converted peripherally to oestrone in women at high risk of breast cancer. Thus Bulbrook's observations might be evidence in favour of Siiteri's "oestrone hypothesis".

However, Kirschner et al (1978) found that androstenedione and oestrone production rates were no different in breast cancer patients than in controls, and that the conversion of androstenedione to oestrone was not abnormal in women with breast cancer. They also found that approximately 30% of oestrone in postmenopausal women with breast cancer comes from sources other than androstenedione, compared with less than 10% in normal women. Nevertheless, in a recent report (Wang et al 1979), Bulbrook's group found that plasma androgens correlated with urinary androgens, and they felt that urinary androgen levels truly reflected adrenal androgen production, which therefore, they contended, is low in high-risk women.

Adams (1978) suggested that urinary androgens may be low because DHEA is metabolised by an alternative pathway, not to oestrone but to another adnrogen, ADIOL: this androgen has been shown to affect oestrogen receptors in the rat mammary gland.

Although it is suspected that the abnormalities of androgen metabolism may reflect in some way an abnormal oestrogen stimulus to the breast, their significance is far from clear. The picture is further complicated by the finding (Grattarola 1980) of a weak correlation between increased urinary testosterone and breast cancer, in study of ovariectomised patients.

5 Other Hormones

Evidence that prolactin may be involved in the initiation or promotion of breast cancer comes from the findings of Kwa et al

(1974) and Henderson et al (1975) that plasma prolactin levels are raised in the families of patients with breast cancer (though not in patients themselves). Sherman et al (1979) found normal prolactin levels during the menstrual cycle in women with breast cancer. In 1974 reports that breast cancer was associated with reserpine use (Armstrong et al 1974; Heinonen 1974) led to speculation that this drug may affect the breast by stimulating the release of prolactin. However, the association between reserpine and breast cancer has been denied in later investigations (O'Fallon et al 1975). Suggestions of a connection between hypothyroidism and breast cancer remain unconfirmed (Kirschner 1977).

III HYPOTHESIS: THE MENSTRUAL CYCLE AND THE AETIOLOGY OF BREAST CANCER

The importance of parity is clear from the foregoing review. Although a woman's total number of children and duration of breast-feeding are probably not important, the first full-term pregnancy has a significant effect on the subsequent risk of breast cancer. Before first full-term pregnancy the epithelial cells are susceptible to some kind of precancerous change, to which they are less susceptible after this pregnancy. The importance of the age at menarche (the earlier the menarche the higher the risk of breast cancer) suggests that the premalignant influence begins at puberty and continues until first pregnancy. The longer the interval between menarche and first pregnancy, the longer the exposure to this premalignant influence and the higher the risk of subsequent breast cancer.

A A previous hypothesis: the menstrual cycle is harmful to the breast

Short (1974) has suggested that the premalignant influence operating during this time is the hormonal fluctuation of the menstrual cycle itself. His hypothesis was that during the interval between menarche

and first pregnancy, the breast is awaiting the surge of hormones induced by pregnancy, which will complete the development of the gland into a fully functional lactating organ. It might be that the gland which is awaiting this hormonal surge is particularly sensitive to changes in hormonal concentrations, and becomes "stable" only after it has fully developed and then involuted. Thus menstrual cycles before the first full-term pregnancy may represent a "stress" to the immature breast.

Short's hypothesis would explain the importance of the age at menarche and the age at first pregnancy as breast cancer risk factors. The hypothesis would not be incompatible with more detailed hypotheses such as the "oestrone hypothesis" or the "oestriol hypothesis", since these oestrogens are present before the first pregnancy and can act on the breast then. It would explain why breast cancer appears only in a few species, particularly the human, since only in humans and domesticated animals is fertility restricted artificially to produce a long interval between the beginning of reproductive life and the start of first pregnancy. When fertility is unrestricted, first pregnancy follows ovulation fairly quickly, and the breast therefore is subjected to only a few months of hormonal fluctuation while still in its hypothetical "hypersusceptible" state. Short's hypothesis would also help to explain the increased incidence of breast cancer in this country: although girls are no younger at marriage now than they used to be, the age at menarche has fallen steadily during this century to a mean of 13.2 years, at which it now seems to have stabilised (Tanner 1973; Roberts and Dann 1975). This has increased the length of time that the menstrual cycle can act on the breast before first pregnancy.

Short's hypothesis also has the particular attraction of having clinical applicability. If the menstrual cycles before first pregnancy stimulate the breast and those after first pregnancy do not, the stimulus could be removed by abolishing the monthly fluctuations of the cycle - for example, by continuous dosage with the combined oral contraceptive pill. Taking the Pill in a three-monthly cycle rather than a monthly cycle is acceptable to many women (Loudon et al 1977), and might even be a surer method of contraception than allowing hormone levels to fluctuate slightly each month. Even taking the Pill on a conventional monthly regime might protect the breast, although such an effect has not yet been demonstrated epidemiologically.

If in the present study cyclical changes had been found among nulliparae but not among parous women, this hypothesis would have been supported. However, the reverse was the case. Before first pregnancy the breast seems unresponsive to the changes of the menstrual cycle, and after first pregnancy it shows cyclical changes. It therefore appears that the cyclical variations in breast epithelial cell activity are not harmful to the stability of the cells. Indeed, the reverse seems to be the case: the <u>inability</u> of the nulliparous breast to undergo these cyclical changes may be connected to its susceptibility to malignant change.

B A modified hypothesis: the immature breast is less sensitive to progesterone

A new hypothesis can therefore be formulated. Before first pregnancy the breast is an "immature" organ, and it only reaches full maturity after achieving the full histological and functional development at the end of pregnancy. While it is "immature" it is susceptible to

premalignant influences, and the longer the stage of immaturity, the greater is the risk of premalignant changes occurring. After full maturity has been reached, the influence of premalignant factors is much less. The most important aspect of the immaturity of the breast before first pregnancy is its inability to respond to progesterone. Although the immature breast will respond to progesterone if the concentration is high enough, large amounts of the hormone (as achieved during the middle trimester of pregnancy) are required to produce an effect on the epithelial cells. On the other hand, after maturity is reached, the epithelial cells will respond to much smaller concentrations of progesterone.

It is suggested, then, that the hormonal aetiology of breast cancer is in some respects similar to that of endometrial cancer - a parallel that other workers have already suggested (Korenmann 1980a,b). In fact, much interest is now being shown in the idea that progesterone protects against breast cancer, or is a marker for a protective factor (Cole 1980). Possibly progesterone can oppose the carcinogenic effect of oestrogen both on the endometrium and on breast tissue.

As far as the endometrium is concerned, it is now widely thought that malignant change can be produced by prolonged exposure to oestrogen unaccompanied by progesterone. This has been reported to occur with post-menopausal oestrogen therapy (Ziel and Finkle 1975). Although as Feinstein and Horwitz (1978) point out, the risk of exogenous oestrogens causing endometrial cancer may have been exaggerated by bias in diagnosing the condition, this seems not to have been the case. Thomas (1978) summarises eight case-control studies, all but two of which show a strong relationship between exogenous oestrogens and endometrial cancer. Endometrial cancer is also associated with

conditions (such as functioning ovarian tumours) in which oestrogen is produced unaccompanied by progesterone over long periods of time before the menopause (Siiteri 1978).

It is now thought that the apparently carcinogenic effect of oestrogens on the endometrium could be modified by progesterone, and this theory is reflected by modifications of regimes of post-menopausal "hormone replacement therapy" by the addition of a progestogen, in such a way as to mimic the production of progesterone during the luteal phase of the normal cycle (Studd et al 1978). There is now evidence that this addition of progestogens does decrease the incidence of endometrial hyperplasia (Sturdee et al 1978); Craft et al 1978), and may therefore decrease the incidence of endometrial cancer (Paterson et al 1980).

It is now thought that a similar protective effect of progesterone may exist for breast epithelium. The molecular basis for this effect remains unclear, however, and the theory is by no means substantiated. Nevertheless, alternative theories are proving disappointing, and the progesterone theory seems at the moment to be a promising one (Cole 1980; Korenmann 1980a,b), as discussed earlier in this chapter.

The theory that the breast may have a different sensitivity to progesterone before and after first pregnancy has not been put forward before. Taken in conjunction with the progesterone hypothesis, this theory would explain a number of the observations about the epidemiology of breast cancer. In particular, it would explain the importance of the age at menarche and the age at first full-term pregnancy. After the menarche there are a few months of anovulatory cycles, but when ovulation begins, progesterone is

produced regularly during the years between menarche and first pregnancy, in concentrations similar to those in cycles after first pregnancy. However, if before the first pregnancy the breast epithelium is relatively insensitive to progesterone, the end result will be the same as if there were simply a long series of anovulatory cycles between menarche and first pregnancy - the effect of the oestrogen present in the circulation will be "unopposed" by the progesterone, despite the presence of the latter hormone in normal concentrations.

The hypothesis explains why studies have not revealed consistent differences in hormone concentrations between groups of women at different risks of breast cancer (Kirschner 1979). If the difference between the groups is due to some extent to the breast's responsiveness to hormones rather than to their circulating levels, then differences in urinary excretion or plasma concentrations will not necessarily be found. Thus two groups of women with exactly similar patterns of steroid hormones in the circulation could be at different risks because of variation in the effects of the hormones rather than their concentration.

Nevertheless, the hypothesis is not incompatible with other hypotheses, including the "oestrone", the "oestriol" and the "progesterone window" hypotheses. The present hypothesis offers no opinion on which type of oestrogen is important in carcinogenesis. Oestrone, oestradiol or oestriol may be exerting the effect which is unopposed by progesterone. It seems likely, in fact, that the interaction of these hormones is more complex than we suspect at the moment: for example, Wotiz et al (1978), measuring progesterone receptors in the rat, found that synthesis of progesterone receptor can be induced by oestradiol, but

this induction is inhibited by oestriol. Progesterone receptors in human breast tissue remain difficult to measure, and until more is known about them, speculation about hormone interaction at a molecular level at the cell membrane will be hard to substantiate or disprove.

The present hypothesis extends, rather than competes with, theories of the importance of progesterone such as the "inadequate luteal phase" theory. This theory, it is suggested, is only operative after the first full-term pregnancy. If ovulation is infrequent after the first pregnancy, or if only small amounts of progesterone are produced during the luteal phase, the "unopposed" action of oestrogen will continue after the first pregnancy, despite the ability of the breast epithelium to respond to progesterone if present. The inadequate luteal phase before first pregnancy is irrelevant, according to the present hypothesis. There are, however, two possibilities for the inadequate luteal phase before first pregnancy having an effect on breast cancer risk. One is that anovulation will be associated with infertility, and therefore women with low progesterone concentrations will have delayed first pregnancies, thus increasing their risk. The other is that the breast's insensitivity to progesterone may be partial rather than complete before first pregnancy, so that a protective effect of progesterone may be present, though much diminished compared with its effect after first pregnancy.

A recent observation which affects the present hypothesis is the finding that oral contraception may influence breast cancer risk before first full-term pregnancy but not after it (Pike et al 1981; Royal College of General Practitioners 1981). The present hypothesis does not permit any conclusions to be drawn about the role of the Pill in influeencing breast cancer risk. If the breast before first pregnancy

is insensitive to progesterone it will almost certainly be insensitive to progestogens in oral contraceptives. Therefore the question as to whether or not oral contraceptives influence the risk of breast cancer becomes a question of whether exogenous oestrogen as provided by the Pill is more, or less, carcinogenic than endogenous oestrogen from a woman's own ovaries. Since the nature of the carcinogenic effect of oestrogens is still not understood, this question is impossible to answer on theoretical grounds, and the answer will have to await a prolonged period of observation of women taking exogenous oestrogens. However, the present indications that the Pill may have different effects among parous and nulliparous women (though as yet these indications remain unconfirmed) support the present hypothesis to the extent of indicating that the hormone sensitivity of the breast, as far as carcinogenesis is concerned, may be different before and after first pregnancy.

The present hypothesis does not explain all epidemiological observations regarding breast cancer, which may be a disease with several causative factors. It does not, for example, explain racial or geographical variations. Although the decrease of the age at menarche would allow the hypothesis to explain the increasing incidence of the disease over recent years, this will probably not be confirmed until another 40 years have passed, when the present levelling off of the fall in the age at menarche may be reflected by a levelling off of the increase in breast cancer incidence.

The present hypothesis draws attention to the response of the breast epithelium to hormones. Much attention has been focused on the levels of hormones in plasma and urine as indicators of risk of breast cancer, but it may be that attention would be better concentrated on the breast

epithelium itself. The relevance of the hypothesis to clinical practice and future research will be outlined in the final section.

C Clinical relevance and future prospects

To test any hypothesis on the causation of breast cancer is difficult because of the probable long interval between cancer initiation and the presentation of the disease. Because several factors may be involved, large numbers of women have to be studied. Nevertheless, the present hypothesis should be capable of being proved or disproved within the foreseeable future. As methods improve further information on the response of normal breast tissue to endogenous hormones should become available. For example, refinements are to be expected in methods of detecting steroid receptors - and progesterone receptors will be of particular interest - and of detecting milk-specific proteins such as casein or α-lactalbumin. Such investigations will eventually confirm or refute the hypothesis that before the first full-term pregnancy the breast is "immature" functionally, and relatively insensitive to progesterone.

If such investigations support the present hypothesis, it will indicate possible methods of prevention of breast cancer, although in the short term the prospects for preventing the disease seem as distant as ever. If initiation of a precancerous process is inseparable from functional immaturity of the gland, it is difficult to see how the process can be prevented without the occurrence of a full-term pregnancy soon after the menarche. Possibly in the future methods will be developed of perhaps inducing the synthesis of receptor proteins, but this prospect is a distant one.

Nevertheless, the hypothesis, if correct, will indicate the direction which should be taken by research into contraception. It appears inevitable that women will in general wish to continue the present pattern of childbearing, with a relatively long interval between menarche and first pregnancy. If anything, it seems likely that this interval will become longer rather than shorter. At present the method of contraception used to delay first pregnancy is usually the oral contraceptive. Already doubts are being raised about the effect of the oral contraceptive on breast cancer risk, and whether or not these fears are well-founded, it already seems likely that the oral contraceptive is not protective against breast cancer.

The ideal contraceptive, as Short (1979) has pointed out, will have positive effects on health, rather than merely minimal ill-effects. If the present hypothesis is correct, then a contraceptive method which provides exogenous oestrogen will continue to initiate pre-cancerous change, even though progestogen is also provided. Therefore research should be directed at suppressing ovarian activity without providing exogenous oestrogen. Baird (1976) has suggested that it ought to be possible to develop a contraceptive which suppresses the hypothalamic-pituitary-ovarian axis without adminstering oestrogen - for example, it could be achieved by mimicking the increased hypothalamic sensitivity to oestrogens which is the normal condition throughout childhood.

The idea of decreasing the interval between menarche and first pregnancy by attempting to re-create the premenarcheal endocrine status may seem fanciful and is certainly not immediately feasible. Nevertheless, if oral contraceptive use before first pregnancy does prove to increase the risk of breast cancer, young women are going to stop taking the

Pill, and pressure will increase for alternative methods to be developed. There is a need, therefore, for further work in two areas: first to examine in more detail the effects of oestrogen and progesterone on the mammary epithelium of nulliparous women (and to accumulate further evidence for or against the hypothesis outlined here); and second, to develop contraceptive methods which will avoid the administration of these steroids to young women, and diminish the risk of breast cancer initiation.

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APPENDICES

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Appendix 1:
Data Sheet.

BREAST HISTOLOGY PROJECT

I. HISTORY

A Personal Data

Name

Date of Birth / / - age: G.P.

Age at first period (sure?)

Date of first birth

Parity Breast-feeding

B Menstrual History

L.M.P. Pre-menstrual symptoms

Cycle Breast tenderness

Regularity - before first pregnancy

- recently

C Contraceptive History

Pill - dates

D Date of First Consultation

E Date of next period

II. BIOPSY

i) Date

ii) Site - quadrant distance from nipple

iii) Diagnosis

iv) Plasma progesterone

III. TISSUE

Formalin

Glutaraldehyde

Frozen

Incubation

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Changes in breast volume during normal menstrual cycle and after oral contraceptives

MILLIGAN, J O DRIFE, R V SHORT

itish Medical Journal, 1975, **4**, 494-496

ummary

he volume of the left and right breasts was measured aily in four nulliparous women during normal mens- ual cycles and after the use of oral contraceptives. reast volume increased significantly in the second half f both normal and contraceptive-controlled cycles. The ean total change in volume throughout the cycle was 0 ml under natural conditions and 66 ml on oral ntraceptives.

troduction

he human breast seems to be uniquely sensitive to ovarian eroids. Man is the only primate in which gross morphological east development is completed at puberty; in all other imates the mammary gland develops only as a consequence of e more profound hormonal changes that accompany preg- ncy.

Not surprisingly, therefore, many women report breast anges during the normal menstrual cycle, with a feeling of llness and a tingling sensation immediately before menstrua- on.[1] Women taking oral contraceptives also seem to experience milar breast symptoms.[2] It has been claimed that there are so pronounced changes in breast volume during the normal enstrual cycle, with maximum values occurring in the week fore menstruation.[3] No attempt has been made, however, to udy changes in breast volume in women on oral contra- ptives. We therefore decided to obtain accurate quantitative formation on the nature and extent of breast volume changes nulliparous women during the course of normal and contra- ptive-controlled cycles.

ubjects and methods

Four healthy nulliparous women, all aged 21 years, were studied r three months.

Case 1—Before the study this woman had been taking mestranol 50 µg us norethisterone 1 mg (Norinyl 1) daily in 21-day cycles which were each llowed by seven tablet-free days. Observations were begun during the cond normal cycle (21 days) after she had stopped taking the contraceptive. the next cycle she again took Norinyl 1.

Case 2—This woman had been taking mestranol 50 µg plus norethisterone mg (Ortho-Novin 1/50) daily in 21-day cycles (followed by seven days of treatment), and observations were begun on the sixth cycle. This was llowed by a normal ovulatory cycle (32 days) and then another contra- ptive-controlled cycle.

Case 3—This woman was observed during three consecutive contraceptive- ntrolled cycles. She was taking ethinyloestradiol 50 µg plus norethisterone etate 3 mg (Gynovlar 21) daily in 21-day cycles followed by seven tablet- ee days.

Case 4—This woman was not taking oral contraceptives and she was udied for one normal 29-day cycle.

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These observations provided data on three complete normal cycles and six complete contraceptive-controlled cycles. In one woman (case 2) the occurrence of ovulation during the normal cycle was confirmed by serial progesterone determinations.

Volume measurement technique—A glass mixing bowl 7 inches (17·8 cm) in diameter standing inside a container on the floor was filled to the brim with water. The woman, kneeling on the floor, lowered one breast into the bowl, thus displacing water into the surrounding container. The volume of water displaced was measured in a 1-litre graduated cylinder. Variability due to postural changes was controlled by marking positions for the container, hands, knees, and elbows on a sheet of plastic. Each woman made three consecutive measurements on each breast every day at the same time, using water of about the same temperature. Repeated measurements were also taken from one woman throughout one day, and the results related to previous posture. A series of consecutive measurements was also made over 40 minutes on the right breast of one woman at water temperature ranging from 45-15 C.

Results

Reliability of technique—A measure of the precision of the technique was obtained from the correlation between measurements made on left and right breasts for each day of the cycle. The correlation coefficient was highly significant ($P<0.001$) for all individual cycles (table I). The "error" in the method was calculated by expressing the variation between consecutive measurements made on one breast on any one day as a percentage of the total change in volume during the cycle. Table I shows the error for each subject expressed as a coefficient of variation. Results of experiments to show the effects of previous posture and temperature on breast volume are given in tables II and III. There was a significant change in volume with posture ($P<0.001$) and a significant decrease in volume with decreasing water temperature ($0.05>P>0.02$).

TABLE I—*Correlation between measurements on left and right breasts, showing precision of technique*

Case No	Correlation coefficient between measurements on left and right breasts	Overall coefficient of variation
1	0·859	2·1
2	0·728	8·5
3	0·865	3·2
4	0·889	5·4

TABLE II—*Variation in breast volume after consecutive changes in posture*

	Horizontal for:		Vertical for:	
	4 hours	11·5 hours	4 hours	11 5 hours
Right breast (ml) ..	523	552	532	562
Left breast (ml) ..	530	588	543	600

TABLE III—*Variation in breast volume with consecutive decreases in water temperature*

Temperature (C) ..	45	40	35	30	25	20	15
Breast volume (ml)	542	564	571	514	525	505	518

Cyclical changes—Breast volume increased significantly during the second half of normal and contraceptive-controlled menstrual cycles ($P<0.001$; fig 1), although there was no consistent difference in total

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volume change between normal and contraceptive-controlled cycles (figs 2 and 3). The pattern of volume change seemed to be different within one woman under different hormonal conditions (see fig 1). In the normal ovulatory cycle the smaller breast volumes were found on days 9-17, with a steep rise until day 25 and a subsequent gradual

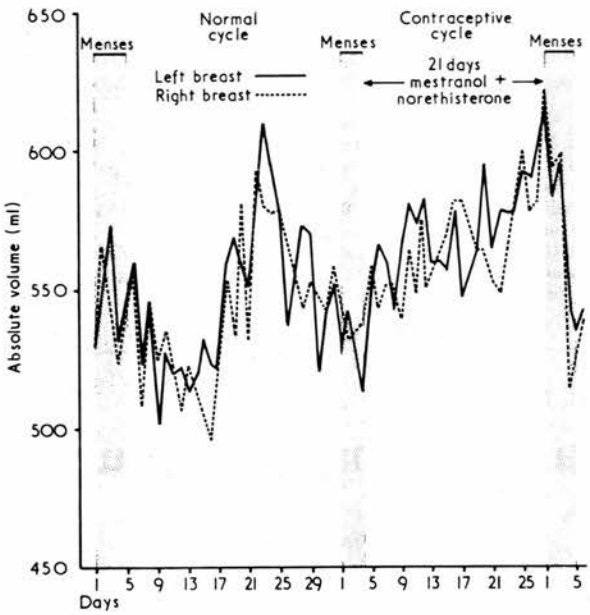

FIG 1—Case 2. Absolute volume changes with time throughout normal cycle and subsequent contraceptive-controlled cycle.

decrease up to and during the menses. In the contraceptive-controlled cycle breast volumes rose steadily throughout the 21 days of steroid treatment, reaching a peak 4 days after completion of the course and dropping rapidly during the period of withdrawal bleeding.

Although the decrease in breast volume in contraceptive-controlled cycles started on different days in different women, in every case the main decrease occurred during the week when the pill was not taken. Breast volumes increased again with the start of a new course of steroid treatment (fig 3). Minimum volumes occurred about a week earlier in contraceptive-controlled cycles than in normal menstrual cycles. The pattern of breast volume changes in normal cycles (fig 2) followed closely the incidence of subjective feelings of tenderness and swelling of the breasts reported by McCance.[1]

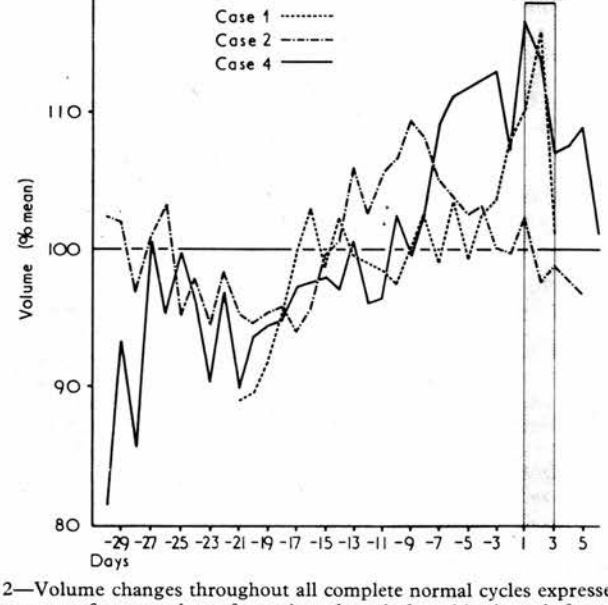

FIG 2—Volume changes throughout all complete normal cycles expressed as percentages of mean volume for each cycle and plotted backwards from first day of menses.

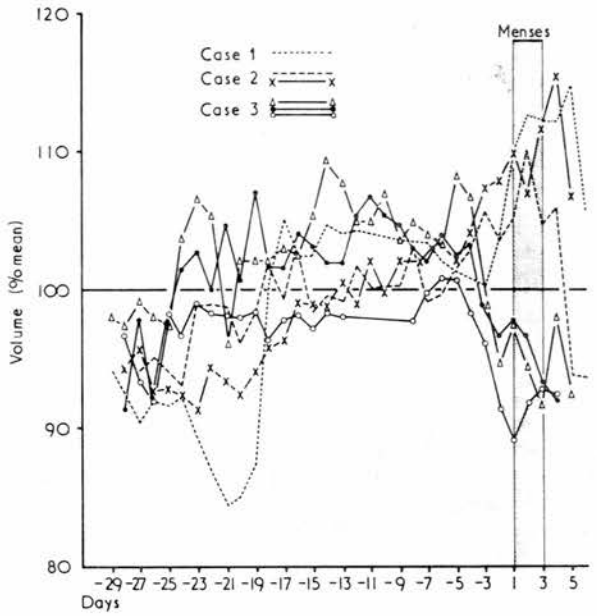

FIG 3—Volume changes throughout all complete contraceptive-controll cycles expressed as percentages of mean volume for each cycle and plott backwards from first day of menses.

Discussion

Others have attempted to measure changes in human brea volume during the normal menstrual cycle by planimetr measurements of breast radiographs[4] or a water displaceme technique.[5] The most detailed study is that of Ingleby,[3] wh made weekly plaster casts of the breasts and then weighed th wax impressions made from these casts. She found the smalle breast volumes in the follicular plase of the cycle in seven o of nine women, with an 8-44% increase above minimum valu during the second half of the cycle.

Masters and Johnson[6] reported a 20-25% increase in brea volume during intense sexual excitement, which they attribute to deep vasocongestion; they do not make clear, howeve whether these changes were objective measurements or mere subjective assessments. Hytten[7] measured breast volum changes during pregnancy in 11 women by a water displaceme technique. He found that the breasts had often attained the maximum volume by the end of the second trimester. Even nullipara there was no significant relation between initial brea size and the degree of enlargement, which might exceed 100% but there was a highly significant correlation between the degr of enlargement and the subsequent milk yield.

Our findings confirm and extend these earlier observation on cyclic changes and provide the first information available o the effects of the contraceptive pill. The mean total change natural cycles was 100 ml while the mean total change for a contraceptive-controlled cycles was 66 ml, and the pattern change during normal and contraceptive-controlled cycles w broadly similar (see figs 2 and 3). The high correlation coefficien obtained by each woman for measurements on her left and righ breasts gives some indication of the reliability of this techniqu of measurement.

The fact that a considerable degree of histological[8] an morphological[9] breast development occurs in girls before th menarche is perhaps the strongest argument for believing th this development is oestrogen dependent. Subsequent chang in volume during the menstrual cycle might be a result hormonally controlled vascular and lymphatic changes[10] structural changes in the intralobular stroma[11] and alveola epithelium[21]. In the rhesus monkey[13] alveolar and lobular enlarg ment occurs only in the second half of ovulatory cycles,[3] whic suggests that this is specifically a progestational change.

There is circumstantial evidence to suggest that a repeated ccession of menstrual cycles before the first pregnancy may en be harmful to the breast[14]; recent epidemiological evidence ows that the risk of breast cancer increases with time elapsed om menarche to first pregnancy.[15 16] Such considerations ghlight the importance of a fuller understanding of the changes xing place in the breast during the normal menstrual cycle and ter the use of oral contraceptives.

We thank those women who generously volunteered to take part in is study and are grateful to Dr C S Corker and Mr R Sharpe for tistical advice.

eferences

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Appendix 3

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The Aetiology of Mammary Cancer in Man and Animals

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SYNOPSIS

In order to understand the aetiology of mammary cancer, it is first necessary to have a clear understanding of the physiology of normal mammary development and lactation. The species with the highest spontaneous incidence of mammary cancer are man, the laboratory rat and mouse, and the domestic dog and cat. Mammary cancer is relatively uncommon in primates, even when kept in captivity, and virtually unknown in the cow, which has nevertheless been selected for large udder size and high milk yield for many generations. Why should this be so?

Part of the answer may lie in the fact that mammary cancer is most common in those species that have been ordained by man to spend the greater part of their reproductive lives in the non-pregnant state, undergoing a succession of regular oestrous or menstrual cycles. Another part of the answer may lie in species differences in the hormonal sensitivity of the mammary gland itself. For example, the human breast is unquestionably the most oestrogen-sensitive of all the primates, since it becomes fully developed anatomically at the time of puberty, well in advance of the first ovulation or the first pregnancy. This is not the case in any other primate. The aetiological factors known to predispose to breast cancer in women include early age at menarche, late age at first pregnancy, and late age at menopause. This suggests that it may be harmful to the breasts to be exposed to a succession of menstrual cycles, with high oestrogen levels in the follicular phase unopposed by the protective effect of progesterone.

Absence of pregnancy is also a major aetiological factor in the rat and cat, but is of less importance in the dog. This could be explained by species differences in the endocrinology of the oestrous cycle.

INTRODUCTION

All the epidemiological evidence points to a major role of steroid hormones in the development of mammary cancer; witness for example the low incidence in males, and the protective effect of ovariectomy in females. Although genetical predisposition, infectious agents and chemical carcinogens are also important, especially for the experimental induction of mammary tumours in laboratory animals, the object of this paper

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is to review the hormonal factors that might be involved in the aetiology of the condition.

Before we can begin to appreciate the abnormal, it is first necessary to have a clear understanding of the physiology of normal mammary development and lactation. This may help us to understand why it is that spontaneously occurring mammary tumours are very common in women (cumulative risk, one woman in 20 by the age of 70 in Britain; Doll, 1975), but rare in all other primates (Seibold & Wolf, 1973; Appleby, Keymer & Hime, 1974), very common in dogs (Schneider, 1970), fairly common in cats (Dorn, Taylor, Schneider, Hibbard & Klauber, 1968), and yet virtually unknown in horses and cattle (Priester & Mantel, 1971) (see Figs 1 and 2). At first sight there is no obvious explanation to account for such marked differences in incidence rates between the various species.

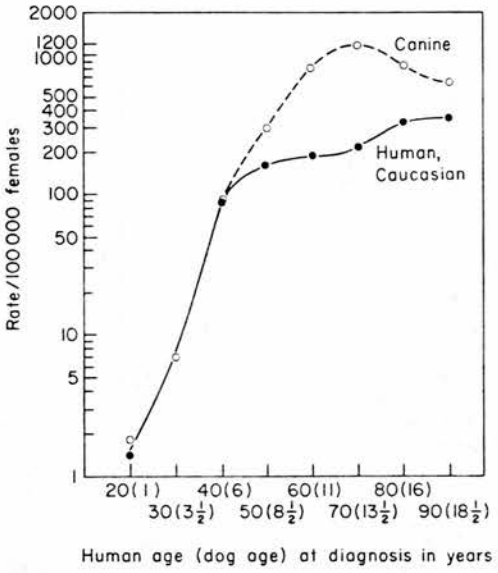

FIG. 1. Annual age-specific incidence rates for human and canine mammary cancer. From Schneider (1970).

Although trite, it is nevertheless necessary to remind ourselves that the mammary gland has been specifically designed by evolution to succour the newborn young. Evolution has also always operated to maximize fertility; pregnancy therefore followed hard on the heels of puberty, and the mammary gland became a functional organ soon after the commencement of an animal's reproductive life. It is interesting that man has chosen to suppress this reproductive potential in only a few selected species: his wife, his domestic pets, the cat and dog, and his laboratory animals. In his farm animals on the other hand, fertility has always been at a premium in

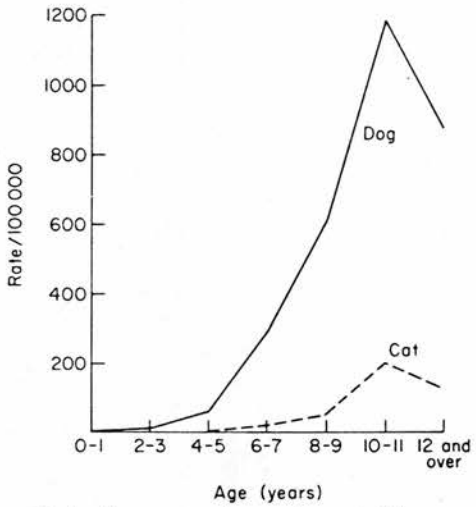

FIG. 2. Annual age-specific incidence rates for canine and feline mammary cancer. From Dorn *et al.* (1968).

order to achieve maximal production of milk or offspring. Could the high incidence of mammary cancer in some species be related to man's self-imposed infertility, resulting in the exposure of the mammary glands to an increased number of sterile oestrous or menstrual cycles before they achieve their ultimate developmental goal of lactation?

Cancer of the mammary gland during the reproductive years would place an individual at a severe disadvantage in evolutionary terms, whereas if the same fate were to befall the individual after reproduction had ceased, it would be beyond the reach of natural selection, and of no significance for the survival of the species. It is therefore interesting to remember that we are the only species in which the female undergoes a true menopause, and the peak incidence of breast cancer is in the post-menopausal years (see Fig. 1).

If we are to attempt to analyse possible endocrine factors that could account for the marked species differences in mammary cancer incidence rates, it is essential to take into account the major differences that are known to occur between species in the hormonal control of mammary development and lactation. These have received scant attention in the past from cancer epidemiologists; for example, Schneider (1970) states that "one event common to man and dog is the same reproductive cycle in the female". Yet an appreciation of these differences must form the basis for any comparative epidemiological study, or any attempt to develop an animal model for human breast cancer. It therefore seems desirable to divide the species up into a number of different lactational categories before attempting to analyse the role of endocrine factors in the genesis of mammary cancer.

THE SIMPLEST SITUATION: MAMMARY DEVELOPMENT AND LACTATION OCCUR AT PUBERTY, AND ARE INDEPENDENT OF PREGNANCY

The most striking examples of this situation are seen amongst the marsupials, of which the red kangaroo (*Megaleia rufa*) will serve as an appropriate case in point.

Male red kangaroos have no pouch and no teats. The four teats first become apparent in the pouch of the female at the time of puberty (Sharman & Calaby, 1964). The length of the oestrous cycle (35 days) is slightly longer than the length of pregnancy (33 days); all the evidence suggests that the hormonal changes during the cycle are identical to those of pregnancy itself, so there has been no need to develop any endocrine mechanism for the maternal recognition of pregnancy (Sharman, 1970). Therefore it is hardly surprising to find that it is unnecessary for female red kangaroos to mate and become pregnant in order to lactate. Sharman & Calaby (1964) successfully removed a newborn joey less than one day old from the teat in its mother's pouch and transferred it to the teat of a virgin, unmated foster mother three days before she herself was due to return to oestrus. The foster mother apparently lactated normally, since the joey had a normal growth rate throughout the duration of its pouch life.

These observations on marsupials may seem a far cry from the aetiology of mammary cancer, especially since there are no reports of such tumours in marsupials. However, the point is made that in some species lactation is normally initiated by the hormonal changes of the oestrous cycle, and no additional endocrine stimulus of a pregnancy is required.

Amongst the eutherian mammals, the domestic dog (*Canis familiaris*) and the ferret (*Mustela furo*) appear to have a pattern of mammary development that is very similar to that of the marsupials. The bitch is a spontaneous ovulator and has a gestation length of about 60 days; she normally comes into oestrus twice a year. The lifespan and secretory activity of the corpora lutea of pseudopregnancy are very similar to those of pregnancy (Jones, Boyns, Cameron, Bell, Christie & Parkes, 1973), suggesting that once again there has been no need for the maternal recognition of pregnancy, and hence no need for the placenta to develop an endocrine function. Thus in any unmated bitch, oestrus is invariably followed by ovulation and the formation of corpora lutea of pseudopregnancy, which persist for about 60 days. During the course of the first pseudopregnancy the mammary glands of the virgin bitch begin to develop and at the end of the pseudopregnancy she may lactate copiously (Asdell, 1964).

Much confusion has arisen in the literature over the misuse of the term "pseudopregnancy" in the bitch. It has tended to lose its scientific connotation (an extended luteal phase in the absence of pregnancy), and has

become a subjective description of the animal's behaviour following oestrus. Thus pseudopregnancy may be described as "mild", "moderate" or "severe", depending on the degree of mammary enlargement noticed by the owner. In "severe" cases, the virgin bitch may even go so far as to make a nest and retrieve and "mother" some inanimate object, whilst lactating profusely.

If the endocrine changes of pregnancy in the bitch are so similar to those of pseudopregnancy, we might expect to find little difference in the mammary cancer incidence rates between mated and unmated animals. This still seems to be a point of some dispute. On the one hand, Uberreiter (1966) stated that pregnancy inhibited the growth of mammary tumours while pesudopregnancy stimulated it, and Andersen (1965 and pers. comm.), in a lifespan study of 354 female beagles, reported that about 50–60% of the unmated animals had developed clinical signs of mammary tumours by the age of ten, whereas only 10% of the parous animals had developed tumours. However, in a carefully executed retrospective case-control study, Schneider, Dorn & Taylor (1969) failed to find any association between pseudopregnancy and mammary cancer risk. Parous females were neither over- nor under-represented in the 93 animals with malignant mammary tumours, nor did there appear to be any effect of age at birth of the first litter, litter number, litter size, or total number of offspring per female. In another careful case-control study, Fidler, Abt & Brodey (1967) even reported that tumour-bearing bitches had significantly fewer episodes of pseudopregnancy than controls.

Further research will be required to resolve this point. In view of the human evidence (see below), it may be the time elapsed to first pregnancy, rather than the total number of pseudopregnancies or pregnancies, that is the critical determinant of the mammary cancer incidence rate in the bitch. From the evidence to date, all that can be said is that there is some suggestion that nulliparous bitches are more likely to develop mammary cancer than their parous counterparts.

In contrast to this somewhat equivocal situation there is excellent evidence to show that early ovariectomy of the bitch has a most pronounced sparing effect on the subsequent likelihood of developing mammary cancer. Schneider *et al.* (1969) showed that bitches that were ovariectomized before their first oestrus had only 0·5% of the mammary cancer risk of intact bitches. If ovariectomy was delayed until after the first oestrus the risk increased to 8%, whereas animals that had two or more oestrous cycles before ovariectomy had 26% of the risk of intact controls. Within this latter group, animals ovariectomized before the age of 2·5 years had a much lower risk than animals ovariectomized after 2·5 years. This evidence would all suggest that mammary cancer is predetermined by the time a bitch is approximately 2·5 years old, and ovarian hormones are crucially important; there is clearly a time-lag of several years between sensitisation and tumour development. Once the tumour is established, however, ovariectomy appears to have no effect on the subsequent course

of the disease. As to which ovarian hormones are the culprits, and how they are implicated, this must remain a matter of speculation until some definitive experimental evidence is forthcoming. Although it is fashionable to implicate oestrogens, Jabara (1962) failed to induce mammary tumours in bitches given large doses of diethylstilboestrol, although this treatment did give a high incidence of ovarian tumours; perhaps the animals were not kept for long enough for mammary tumours to become apparent. The beagle bitch has also become notorious with the drug regulatory agencies for producing benign mammary tumours when treated chronically with synthetic progestagens (Nelson, Weikel & Reno, 1973).

Since the majority of mammary cancers in dogs, as in women, are adenocarcinomas (Schneider, 1970), and since the two species show very similar age-specific incidence rates when their ages are adjusted for differences in absolute lifespan (see Fig. 1), it has often been suggested that the dog is the most appropriate animal model for the study of human breast cancer. The great differences in the hormonal control of mammary development between the two species, to say nothing of the profound endocrine differences during the cycle and pregnancy, should make one cautious about making such a naïve assumption. Nothing seems to be known about the spontaneous incidence of mammary cancer in the ferret. Since its reproductive cycle is so similar to that of the dog, it would be worth exploring as a possible experimental animal for mammary cancer studies.

THE INTERMEDIATE SITUATION: MAMMARY DEVELOPMENT AND LACTATION DEPENDENT ON COPULATION

The domestic rabbit (*Oryctolagus cuniculus*) is perhaps the best example of a species in which mammary development is dependent on copulation. The unmated female rabbit is almost constantly in oestrus or sub-oestrus, but there is no mammary development or lactation. Following a sterile mating, ovulation is induced, and this is followed by a pseudopregnancy of about 16 days (the gestation period is 29 days) and mammary development; at the end of pseudopregnancy, when the corpora lutea are beginning to regress, the doe plucks her hair to make a nest, and starts to lactate (Asdell, 1974). The fact that pseudopregnancy is of shorter duration than pregnancy suggests that the placenta does exert some endocrine influence on the mother, although this is clearly not essential for mammary development. Mammary cancer appears to be a rare event in the rabbit, so it is necessary to look at other animals in this group in order to obtain epidemiological evidence about mammary cancer incidence rates.

The domestic cat (*Felis catus*) is a good case in point. The female is an induced ovulator, and in the absence of copulation, the female will undergo a succession of 10-day periods of oestrus every two to three

weeks from the spring to the autumn. Ovulation does not occur, so no corpora lutea are formed, and mammary development does not take place. However, a sterile mating is followed by ovulation and a pseudopregnancy which does not last as long as pregnancy itself (30 v. 63 days; Asdell, 1964). This, together with the fact that ovariectomy late in pregnancy does not result in abortion (Asdell, 1964), suggests that the placenta has begun to develop an endocrine function towards the end of gestation in the cat. The mammary glands are also said to develop during pseudopregnancy, and lactation may occur when the corpora lutea regress (E. C. Amoroso, pers. comm.).

There is good epidemiological evidence from a retrospective case-control study to show that ovariectomy reduces the risk of mammary cancer in cats about sevenfold (Dorn *et al.*, 1968). In a most extensive uncontrolled investigation, Weijer, Head, Misdorp & Hampe (1972) examined mammary tumours, mostly adenocarcinomas, from 156 cats, of which 114 were intact females, 40 ovariectomized females and two castrated males; the average age of spaying was 5·8 years. One most interesting point was that only 35 of the 114 intact females had ever had kittens.

It is tempting to try and relate these findings to the different endocrine status of the animals. As in the dog, the protective effect of ovariectomy suggests that ovarian steroids are implicated in the aetiology of feline mammary cancer, and it would be interesting to know whether the protective effect is greater the earlier the operation is performed. Normally there are very few opportunities for a cat to become pseudopregnant, since most matings are likely to be fertile. However, there will be a small group of cats that are so jealously guarded by their owners, that although they come into heat regularly, they are never given the opportunity to mate, and so they seldom if ever ovulate. These would appear to be the animals that are most likely to develop mammary cancer. It would require a careful case-control study to establish the point, but it seems possible that in the cat, it is repeated cycles of oestrogenic stimulation of the nulliparous mammary gland that is one of the main endocrine factors predisposing to mammary cancer. It would be particularly interesting to be able to compare the incidence of spontaneous mammary tumours, and the structure of the mammary gland, in three groups of cats, namely those which were never mated, those that had repeated pseudopregnancies following sterile matings, and those that had repeated pregnancies.

In contrast to the rabbit and the cat, the laboratory rat (*Rattus norvegicus*) is a spontaneous ovulator. However, the stimulus of copulation is necessary to activate the corpus lutem and produce a pseudopregnancy. In the absence of mating, the rat will undergo four-day oestrous cycles, and the corpora lutea formed after ovulation secrete very little progesterone for only two days. Following a sterile mating, the corpora lutea are much more active and last for 12 days, whilst following a fertile mating the corpora lutea are fully active and persist for the duration of pregnancy:

22 days (Hashimoto, Henricks, Anderson & Melampy, 1968). At the time of puberty, ovarian oestrogen secretion results in rapid growth of the duct system within the mammary gland. However, alveolar development requires the additional stimulus of pregnancy, and the rat's placenta is known to produce both luteotrophic and mammotrophic hormones (Cowie & Tindal, 1971). There seems to be little information about the degree of mammary development produced by pseudopregnancy.

The reproductive cycle of the mouse (*Mus musculus*) is very similar to that of the rat. In addition to the oestrogen-induced ductular proliferation of the mammary gland at puberty, Faulkin & DeOme (1960) made the interesting observation that the stroma of the mammary gland had an important regulatory action on ductular development.

There is an enormous literature on the genetic, viral and carcinogenic factors that influence the incidence of mammary tumours in rats and mice, and Welsch & Meites (1974) have recently reviewed the literature on the hormonal control of mammary tumours in these two species. There is abundant evidence to show that chronic oestrogen administration increases the incidence of spontaneous mammary tumours, and ovariectomy diminishes it. Similarly, hypophysectomy decreases tumour incidence, whilst multiple pituitary transplantation increases it. It seems reasonable to conclude that oestrogen exerts its carcinogenic effects both by a direct action on the mammary gland, and indirectly by stimulating the secretion of prolactin from the pituitary. But in spite of all this experimental work, few investigators have addressed themselves to the important question of determining how the animal's own reproductive state (repeated oestrous cycles, repeated pseudopregnancies or repeated pregnancies) influences the incidence of mammary tumours. Howell & Mandl (1961) obtained spectacular results by keeping a group of nulliparous rats until they become senile or died; they found a 100% incidence of mammary tumours. A comparable control group, housed with males and hence allowed to become pregnant on every possible occasion, only had a 4% incidence of mammary tumours. It would have been interesting to know the tumour incidence rate in a group undergoing repeated pseudopregnancies.

Although the evidence is admittedly very incomplete, it does seem that in those species in which mammary development and lactation are dependent on copulation, the risk of developing mammary cancer is highest in animals subjected to repeated oestrous cycles in the absence of an ensuing luteal phase. Whether an initial pregnancy, or even a pseudopregnancy, by temporarily transforming the mammary gland into a secretory structure, can reduce the incidence of mammary cancer even though the animal reverts to a succession of oestrous cycles, remains to be determined. Experiments of this nature should be simple and cheap to perform, particularly in rats and mice, and they could yield much valuable information about the stage of mammary development that is most susceptible to neoplastic change following oestrogen exposure. This

might be much more relevant to an understanding of the aetiology of human breast cancer, than all the experiments on "triply operated" rodents (ovariectomized, adrenalectomized and hypophysectomized) given pharmacological doses of exogenous hormones and carcinogenic agents.

THE USUAL SITUATION: MAMMARY DEVELOPMENT AND LACTATION DEPENDENT ON PREGNANCY

Most mammals with which we are familiar fall into this category. They are all, by definition, spontaneous ovulators. The duration of the luteal phase of the oestrous or menstrual cycle is much shorter than the duration of pregnancy, and is seldom referred to as a pseudopregnancy. The extended life of the corpus luteum during pregnancy can be attributed to an endocrine role of the placenta, which may produce a whole variety of hormones. In species like the horse, cow, sheep, goat, pig and guinea-pig, where the life of the corpus luteum is prematurely cut short at the end of the oestrous cycle by the luteolytic action of prostaglandin-$F_{2\alpha}$ secreted from the uterus, the placenta has to exert an initial anti-luteolytic action in order to save the life of the corpus luteum and maintain the pregnancy. In primates, on the other hand, where there is no evidence for a uterine luteolysin, prolongation of luteal life in pregnancy is brought about by the secretion of chorionic gonadotrophin and maybe other luteotrophins from the placenta (Short, 1969). The placenta may also produce a mammotrophic hormone (see the chapter by Forsyth, this volume) and a variety of steroid hormones, including oestrogens and progesterone. These may be manufactured in sufficient quantity to take over the endocrine maintenance of pregnancy, so that ovariectomy of the mother no longer results in abortion.

Thus it can be seen that pregnancy involves major endocrine changes, and it is hardly surprising that nature seems to have used these, rather than the more modest hormonal changes of the shortened oestrous cycle, to initiate lactation. The increased ovarian oestrogen secretion at puberty initiates ductular growth within the mammary gland, but the two- to three-week luteal phase of the oestrous cycle common to all members of this group is barely sufficient to initiate alveolar development; this is well illustrated by the guinea-pig (Turner & Gomez, 1933). If the life of the guinea-pig's corpora lutea is prolonged by hysterectomy, then mammary development occurs and the animal may lactate (Loeb, 1927). If pregnancy is terminated prior to mid-gestation, this is followed by mammary involution, but termination after mid-gestation will initiate lactation (Loeb & Hesselberg, 1917).

The rhesus monkey, *Macaca mulatta*, another member of this group, shows more pronounced mammary changes during the menstrual cycle. Speert (1941, 1948) carried out a most thorough histological investigation

of the mammary gland, and noted that variations *between* individuals at a given stage of the menstrual cycle were greater than those *within* an individual at different stages of the cycle; thus it was essential to study serial biopsies from the same animal. When this was done, there was clear evidence of histological changes during ovular cycles. These changes took the form of lobular enlargement, hyperaemia and alveolar dilatation during the luteal phase, with regression following menstruation. During pregnancy, there was little histological change in the mammary gland from the luteal state for the first two months, but by the third month the lobules had become markedly hypertrophied and the alveoli were enlarged and full of secretion; these changes became even more pronounced with advancing gestation. When abortion was induced on the 36th day of pregnancy, it was followed by mammary regression, but abortion on day 60 resulted in immediate although transitory lactation.

Speert (1948) also obtained some most interesting results on mammary involution following ovariectomy; this produced generalized atrophy of the mammary gland and the appearance of discrete hyperplastic nodules which disappeared following oestrogen or progesterone therapy. He also carried out one of the few detailed investigations of mammary involution following parturition, and showed that this was arrested or even reversed in those animals that started to ovulate again soon after delivery. Finally, Speert studied the effects of chronic administration of large doses of oestrogen or progesterone to intact and castrated monkeys. Depending on the dosage, oestrogen took about a month to produce duct, lobule and alveolar development, although it did not initiate secretory activity. This hypertrophied state could be maintained for the duration of treatment (up to 30 months) with no evidence of spontaneous involution. No mammary tumours were produced in any of these animals, perhaps because they were not kept for long enough, although some showed benign metaplastic changes of the alveolar epithelium. Progesterone was also capable of stimulating alveolar growth and development in castrated animals; it is of interest that a ductal carcinoma has recently been described in one out of six rhesus monkeys given physiological doses of an oral contraceptive (Kirschstein, Rabson & Rusten, 1972).

It would be most exciting if one could begin to relate all this histological information to the aetiology of spontaneously occurring mammary cancer in animals within this group. However, such tumours are exceedingly rare in herbivores (Priester & Mantel, 1971) and sub-human primates (Seibold & Wolf, 1973; Appleby *et al.*, 1974), and perhaps we should be seeking an explanation for this fact. It could be argued that in members of this group the oestrogenic phase of the oestrous or menstrual cycle is followed by an abbreviated although fully-functional luteal phase, which might in some way counteract the effects of repeated oestrogenic stimulation of the mammary gland without producing full alveolar development.

THE HUMAN SITUATION: MAMMARY DEVELOPMENT OCCURS AT PUBERTY, BUT LACTATION IS STILL DEPENDENT ON PREGNANCY

We are the only primate in which the breasts undergo a major anatomical development at the time of puberty. Breast enlargement is in fact the first external sign of impending puberty in girls (Marshall & Tanner, 1974), and the breast can achieve its adult size before the first ovulation, which is usually a year or two after menarche (Short, 1976). The reason is not far to seek; breasts are regarded as erotic in most human societies (Ford & Beach, 1952), and it makes obvious sense to develop the organs of sexual attraction before conception occurs. In contrast, the breasts appear to be devoid of erotic significance in all other primates. Much of this pubertal breast growth is due to stromal development, and the fact that the human breast can respond to the minute amounts of oestrogen secreted by the ovaries at the commencement of puberty suggests that this stromal tissue has become extremely sensitive to oestrogen. Since the stromal tissue of the mouse mammary gland has an important regulatory action on ductal growth (Faulkin & DeOme, 1960), it would be interesting to know if the same was true of human breast stroma.

The development of mammary ducts in women at the time of puberty has been described by Dawson (1934) and Haagensen (1971). Coincident with the stromal development of puberty there is a lengthening and branching of the ductular tree, which is rudimentary in the pre-pubertal girl, and the formation of lobules. Following these initial pubertal changes, the amount of glandular tissue shows wide variations between individuals, and indeed between different areas of the same breast (Foote & Stewart, 1945), and this heterogeneity has bedevilled all attempts to analyse the histological changes in the normal human breast.

Rosenburg (1922) claimed that there was budding of the ductal epithelium in the pre-menstrual phase, with the subsequent disappearance of the acini formed from this proliferation after menstruation had occurred. However, Dieckmann (1925) reanalysed Rosenburg's material, and concluded that the differences he had found were due to the patient's age, rather than to the stage of the menstrual cycle. Foote & Stewart (1945) also concluded that the number of lobules increased with age, but in addition they claimed that the number and size of the alveoli increased prior to menstruation.

Curiously, none of these early workers took into account the parity of the subjects they studied, so there exists no information in the literature about possible changes in breast histology as a result of pregnancy. We have attempted to remedy this deficiency.

Several surgical units in Edinburgh have co-operated to provide us with tissue from 170 women of reproductive age undergoing reduction mammoplasty, or breast biopsy for benign disease. Any subjects who were shown to have histological evidence of malignant breast disease were

excluded from the study. A full reproductive and menstrual history was taken from each subject, and blood was collected for plasma progesterone assay. Each woman was asked to notify us of the date of her next menstrual period.

In the case of mammoplasty, a piece of tissue was taken at random from the specimen removed by the surgeon; in the case of biopsy, a piece of apparently normal tissue was removed through the same incision as the biopsy, but as far away as possible from the abnormal area. The tissue was immediately fixed in 4% neutral buffered formaldehyde, and 5 μm-thickness paraffin-embedded sections were stained and examined.

As in previous studies, we found wide variations within a single breast in the number of lobules per unit area, the number of acini per lobule, and the area of the specimen occupied by lobules; however, the variations between individuals were much greater than those within an individual.

The proportion of glandular tissue varied at random from 0·3% to 29·0% of the area of the section; epithelial height and epithelial cell size were also determined, and an objective assessment was made of the degree of oedema of the intralobular stroma. However, none of these variables showed consistent variations with the stage of the menstrual cycle in either nulliparous or parous women.

The only histological structures that did appear to show a difference between nulliparous and parous women were the vacuolated cells in the basal layer of the alveolar epithelium, as described by Dieckmann (1925) and Bassler (1970). There was a tendency for these cells to be more numerous in nulliparous women, suggesting that the epithelium of the breast might undergo a structural change after the first pregnancy.

Even though histological changes may not be readily apparent in the human breast during the course of the menstrual cycle, structural and functional changes undoubtedly do occur. For example, there are pronounced changes in the total breast volume; nulliparous girls had maximal breast volumes at about the time of menstruation, decreasing by as much as 20% during the early follicular phase of the ensuing cycle; there is even a suggestion that women on the combined oestrogen+progestagen contraceptive pill may show a different pattern of change to women having normal menstrual cycles (Milligan, Drife & Short, 1975). At a functional level, we have found increased IgA synthesis in tissue cultures of human breast during the luteal phase of the cycle in parous women, but not in nullipara (Drife, McClelland, Pryde, Roberts & Smith, 1976), and there are similar changes in DNA synthesis in organ cultures of breast tissue as assessed by the incorporation of tritiated thymidine (Masters, Drife & Scarisbrick, 1977). It seems safe to conclude, from several different lines of evidence, that structural and functional differences do exist between the breasts of nulliparous and parous women.

If the initial growth of the human breast at the time of puberty is for purposes of sexual advertisement only, one would not necessarily expect the volume of the resting breast to give any indication of its potential

functional capacity during lactation. Hytten (1954) in fact showed that there was no correlation between initial breast size at the beginning of pregnancy and subsequent lactational performance, whereas there was a good correlation between the degree of secondary breast enlargement during pregnancy and subsequent milk yield.

How can we begin to relate all this fragmentary evidence about the physiology of the human breast to the epidemiology of human breast cancer? We have many tantalizing clues, but little hard proof of cause-and-effect relationships. Thus it has been known for over two centuries that breast cancer is particularly common in nuns (Doll, 1975) and it is now an established fact that nulliparous women, such as nuns, have a much higher incidence of the disease than parous women (Taylor, Carroll & Lloyd, 1959). The evidence initially suggested that the more pregnancies a women had, the less likely she was to develop breast cancer. However, MacMahon and his colleagues (MacMahon, Cole, Lin, Lowe, Mirra, Ravnihar, Salber, Valaoras & Yuasa, 1970) were able to show in five different areas of the world that it was the age of the mother at the birth of her first full-term infant that determined the risk throughout the rest of her life (Fig. 3). Women who gave birth to their first child before the age of 18 had about one-third the breast cancer risk of those whose first birth was delayed until 35 or later. Indeed, women whose first birth was after the age of 35 had a higher breast cancer risk than nulliparous women. The

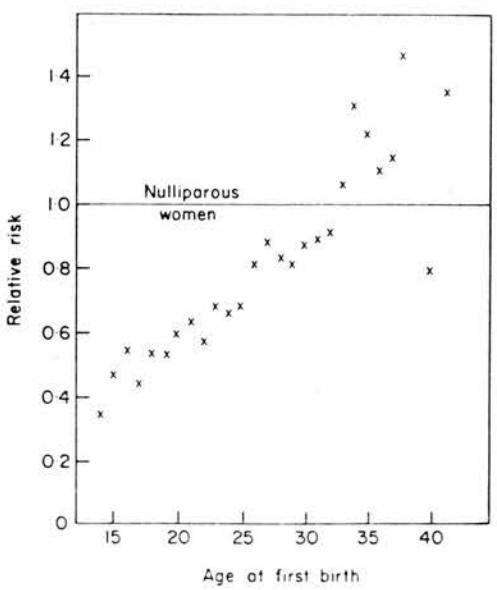

FIG. 3. The relative risk of developing breast cancer in relation to the mother's age at the birth of her first child. The risk in nulliparous women is taken as one. Pregnancy before the age of 35 reduces the risk, and after 35 increases it. From MacMahon, Cole & Brown (1973).

actual number of pregnancies was irrelevant, nor did it matter whether the children were breast or bottle fed. The previous correlation between low cancer incidence and high parity could be explained simply by the fact that the mothers of large families inevitably started reproducing early in life. MacMahon's observations have been confirmed in a large-scale prospective study carried out in New York city (Shapiro, Goldberg, Venet & Strax, 1973), and in a retrospective case-control study in New York State (Lilienfield, Coombs, Bross & Chamberlain, 1975).

The message from MacMahon's work is clear and simple; early first pregnancy protects, whereas late first pregnancy exacerbates. It emphasizes the point that the predisposing factors act early in a woman's reproductive life, but that a pregnancy can protect the breast against these influences. Late first pregnancy may be harmful because it activates a pre-cancerous state that already exists.

Another important factor that has been shown to influence the breast cancer incidence rate is the age of menarche; women with an early menarche have an increased risk (MacMahon, Cole & Brown, 1973; Shapiro *et al.*, 1973). It is well known that the age of menarche varies markedly in different areas of the world, being most advanced in the most developed countries, and most retarded in the poorest (Marshall & Tanner, 1974), whereas the reverse is true for the age at first pregnancy, with the affluent nations having the motivation and the means to defer the birth of the first child, whilst the deprived nations have neither (Short, 1976). It therefore seems significant that the breast cancer incidence rates are highest in the developed countries (MacMahon, Cole & Brown, 1973), which have the longest period of time between menarche and first pregnancy, whereas they are lowest in the developing countries, where the period between these events is the shortest (Fig. 4). Although MacMahon himself rejects it as an explanation, it still seems plausible to imagine that the nulliparous breast may be stressed by a repeated succession of menstrual cycles, whereas following the first pregnancy there is a structural and functional transformation of the breast which protects it from further precancerous change.

It would be fascinating to know the breast cancer incidence rates of women ovariectomized at various ages before and after the onset of puberty, but this information is never likely to become available. However, there is a mass of evidence to show that ovarian hormones do have a major part to play in the genesis of human breast cancer. The classical work of Beatson (1898) showed that ovariectomy could occasionally cause remission of breast cancer in pre-menopausal women. He was led to perform the operation because, he believed, "lactation is at one point perilously near becoming a cancerous process if it is at all arrested", and he asked himself the simple question, "Is cancer of the mamma due to some ovarian initiation, as from some defective steps in the cycle of ovarian changes; and if so, would the cell proliferation be brought to a stand-still . . . were the ovaries to be removed?" There is now abundant

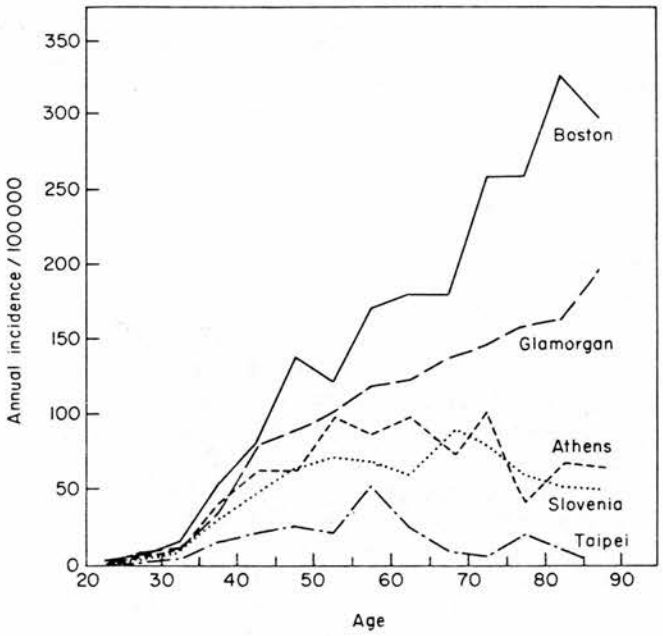

FIG. 4. Age-specific incidence rates of breast cancer in five different areas of the world. Care must be taken in interpreting these data, as there could be "cohort effects" with progressive declines in the age at menarche in the last century. From MacMahon, Cole & Brown (1973).

clinical evidence to show that ovariectomy prior to the menopause reduces the breast cancer risk for the rest of life, and the earlier the operation is performed, the greater the reduction (Trichopoulos, MacMahon & Cole, 1972; MacMahon, Cole & Brown, 1973); ovariectomy before the age of 35 reduces the risk to about a third of that in women having a normal menopause at 45–54, whereas women with a late natural menopause, after 55, have the risk increased by half.

Other aetiological clues are provided by racial differences in breast cancer incidence rates; the Japanese have one of the lowest rates in the world, and yet if Japanese migrate to the United States, their children have incidence rates very similar to those of native Americans (Buell, 1973). This suggests a major influence of environmental factors. There is evidence to suggest that in the early part of this century, Japanese women were probably experiencing a very late menarche and an early first pregnancy, which could account for the low incidence of the disease in Japan in recent decades, whereas American-born Japanese would be expected to conform more to the reproductive norms for the United States, with early menarche and late first birth. Other evidence that also points to a major influence of environmental factors comes from a review of published breast cancer incidence rates in monozygotic and dizygotic

TABLE I

The incidence of malignant breast tumours in female monozygotic and dizygotic twins

	Monozygotic twins		Dizygotic twins	
	One twin affected	Both twins affected	One twin affected	Both twins affected
No. of cases reported in literature	4	4	3	3

Data from Macklin (1940).

twins (Macklin, 1940). These results are summarized in Table I. In spite of the small numbers, and the biases inherent in any survey of the published literature, the 50% non-concordance in monozygotic and also in dizygotic twins would suggest that the environment is much more important than the genotype. For the future, it would be fascinating to see whether differences in reproductive life history could account for these differences between monozygotic twins. However, it is only fair to point out that the female relatives of women with breast cancer are said to have a two- to three-fold increased risk (MacMahon, Cole & Brown, 1973), indicating that genetic predisposition may be quite important. Once again, it would be necessary to establish that this apparent familial trend was not due to a familial similarity in reproductive life histories.

Although all the epidemiological evidence about human breast cancer points to an involvement of the ovaries, it does not tell us which ovarian hormone is likely to be the culprit. Now that many millions of women have been exposed to a combined oestrogen + progestagen oral contraceptive for up to a decade, and many thousands of women have been taking oestrogens in the form of post-menopausal hormone replacement therapy for an even longer period of time, we are beginning to get some answers.

There is general agreement that the combined oral contraceptive has not led to an increased incidence of breast cancer (Arthes, Sartwell & Lewison, 1971; Vessey, Doll & Sutton, 1971, 1972; Boston Collaborative Drug Surveillance Programme, 1973; Royal College of General Practitioners, 1974), although Fasal & Paffenbarger (1975) have suggested that oral contraceptives may hasten the development of pre-existing cancer. However, all the above studies have shown that oral contraceptives do reduce the incidence of benign lesions of the breast. Since women with benign breast lesions have an increased likelihood of subsequently developing a malignant tumour (Davis, Simons & Davis, 1964; Black, Barclay, Cutler, Hankey & Asire, 1972; MacMahon, Cole & Brown, 1973), it is reasonable to hope that the oral contraceptive may ultimately be shown to reduce the incidence of malignant breast disease. We shall have to wait for several more years before women who took the oral contraceptive early in their reproductive years enter the high breast

cancer incidence age group. When they do, it will be particularly interesting to know whether taking "the pill" for an extended period of time in the sensitizing years prior to first pregnancy has had a greater sparing effect than when taken after the first pregnancy. Since the pill contains both an oestrogen and a progestagen, it prevents the breast tissue from being exposed to oestrogen alone, whereas during the first half of the normal menstrual cycle, the ovaries are secreting oestrogen and little or no progesterone.

It is only very recently that reliable evidence has become available about breast cancer incidence rates in post-menopausal women on oestrogen replacement therapy (Hoover, Gray, Cole & MacMahon, 1976). The risk of developing breast cancer is increased by this therapy, particularly 10 years or more after first exposure; 15 years after the start of treatment, the risk was doubled. Furthermore, this oestrogen replacement therapy appears to cancel out the normal protective effects of multiparity and oophorectomy, and enhance the chance of malignancy in those with benign breast disease.

All this evidence leaves one with the strong impression that oestrogens may be involved in the genesis of human mammary cancer, and that the breast is in a particularly vulnerable state in the period between menarche and first pregnancy. If we could discover more about the functional changes that the breast undergoes during this time, we might come much closer to an understanding of how breast cancer is caused. Since it seems likely that we shall be using steroid hormones on an ever-increasing scale to regulate human reproduction, it would be comforting to think that we knew enough to use them judiciously. They might enable us to prevent not only pregnancy, but breast cancer as well.

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NOTE ADDED IN PROOF

Recent evidence suggests that lactation itself may have a cancer-sparing effect on the human breast. Ing, Ho & Petrakis (1977) studied a group of Chinese women in Hong Kong who traditionally only fed their babies from one breast. They found that cancer developed only in the unsuckled breast in 79% of such women when postmenopausal, a significantly different distribution from that seen in the normal Chinese population in Hong Kong.

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Appendix 4: Commentaries in Research in Breast Disease 1: 1-23.

Evolution, Menstruation, and Breast Cancer

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Darwinian man, though well behaved,
At best is only a monkey shaved.

(Princess Ida)

W. S. Gilbert had a better grasp of scanning than of evolution. There are other differences between humans and monkeys besides their behavior and their hairiness. For example, in our own species breast cancer is the commonest malignant disease that affects the female, but female monkeys – although they menstruate and suckle their young – rarely suffer from the disease [Seibold and Wolf, 1973].

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Our progress from jungle treetop to city street has been a retrograde step as far as the breast is concerned, and in this article I shall discuss some of the stresses to which the human breast is uniquely subjected. Such stresses may go some way towards explaining why malignant disease is so common in the human breast.

I. EVOLUTIONARY ASPECTS

A. Evolution and Menstruation

There are marked endocrinologic differences between the menstrual cycles of primates and the estrus cycles of other mammals [Short and Drife, 1977], but the menstrual cycles of the human and the monkey are basically similar. The mammary glands of both humans and monkeys are therefore subjected to similar monthly fluctuations in the plasma levels of ovarian steroid hormones. One important difference, however, is that the human is subjected to many more of these than is the monkey.

Women nowadays tend to accept their several decades of menstrual cycles as normal and physiologic, but it has been argued [Short, 1976] that evolution has prepared the human female, not for a long series of menstrual cycles, but for a long series of pregnancies. Until the twentieth century, large families were normal, and of course contraception was practically unknown. In the past – and indeed even today in developing countries – it was necessary for a couple to produce up to 10–12 pregnancies in order to have a fighting chance that two or three of their offspring would survive beyond childhood to marry and reproduce. The remaining offspring would be carried off by stillbirth, neonatal death, or epidemics of infectious disease in childhood.

The development of artificial feeding is another relatively recent change causing the modern woman to experience more menstrual cycles than her counterpart of even a century ago. Lactation, as well as delaying the return of regular menstruation, also acts as a natural contraceptive, and so pregnancies are spaced out in a physiologic way. There is evidence that if a woman is undernourished, the return of lactation is further delayed, and so those women least fit to undertake another pregnancy would enjoy the surest contraception. Short [1976] suggests that the "natural" cycle for the female of our species is a cycle of pregnancy and lactation, with only a few menstrual cycles between the end of lactation and the start of the next pregnancy. If lactation lasts for over a year, such a cycle would result in fewer than ten pregnancies between a woman's late teens and her early forties, and during all this time a fertile woman would experience only a few dozen menstrual cycles.

Just as important as the total number of menstrual cycles experienced by a woman is the phase of her life at which she experiences them. The age at menarche has been steadily falling during this century (possibly as a result of improvement in nutrition), though in Europe this decrease appears to have stopped now around the age of 13 [Tanner, 1973]. During the nineteenth century, when the average age of the menarche was around 18, the usual age of marriage was much the same as it is nowadays. The first few cycles after the menarche are anovulatory, and so the first ovulatory cycles would occur around the time that a girl was married. In the absence of contraception, conception would normally occur very soon after marriage, and so the average woman of the nineteenth century would experience very few of the hormonal fluctuations of the menstrual cycle before she became pregnant. This is a particularly important point in view of the hypothesis to be developed later in this article.

The difference between the conditions I have just described and those prevailing today hardly needs emphasizing. The average age of the menarche in Great Britain today has stabilized at 13.2 years, and the average age of the menopause is 50.8 years. The average family size is 2.4 children. Breast feeding remains the exception rather than the rule. The average woman is, therefore, likely to experience a total of over thirty years of menstrual cycles. Though individuals vary greatly, for most women at least five of these years–and for many women, more than ten of these years – will be before the first full-term pregnancy occurs. This situation has arisen partly as a result of evolution, which has made the human female a spontaneous ovulator, and partly as a result of modern civilized life, which has made it fashionable and indeed necessary to impose artificial restraints on natural fertility.

Unfortunately neither evolution nor civilization can be relied upon to have completely beneficial effects on the individual. The process of evolution aims at adaptation of the species as a whole, and if an adaptation results in harmful effects on the individual after the reproductive phase of that individual's life, then there will be no evolutionary selection against such an adaptation. In other words, if a relentless succession of menstrual cycles makes a woman a more efficient ovulator, they will be an evolutionary success, and this evolutionary success will not be jeopardized if the cumulative effect of the menstrual cycles is to increase the incidence of disease over the age of 45.

As far as our civilized life-style is concerned, the side effects of this are only just beginning to become apparent. For example, although the side effects of contraceptives are being monitored with care in various centers throughout the world, the side effects of infertility per se have been neglected – the assumption being that there are none. Some degree of voluntary infertility is, and will probably continue to be, necessary for the survival of our species, but the effects of this infertility on the individual are not necessarily beneficial. For example, it is associated with gynecologic disorders such as fibroids and endometriosis, and the evidence for a connection between infertility and breast disease will be discussed below (Section III).

B. Evolution and the Breast

1. The stroma. The human breast is unique among mammalian mammary glands in being a prominent organ during the nonpregnant and nonlactating state – the "resting" state, as I shall call it. During this time its prominence is produced by a mass of fatty tissue and stroma, with only a minor contribution from glandular tissue. During pregnancy the glandular tissue hypertrophies, until at the time of lactation the breast consists mainly of secretory acini, with only a little supporting stroma – as in most other functioning glands [Dawson, 1934]. If the stroma is replaced by glandular tissue during lactation, what is its function? It seems unnecessary for lactation: In the Great Apes the breast is completely flat and only becomes prominent towards the end of pregnancy when the glandular tissue hypertrophies. In these species the adequate functioning of the organ during lactation does not require a large quantity of supporting stroma, and it is worth noting that in the human, milk yield during lactation is related not to the nonpregnant size of the breast, but to the increase in size during pregnancy [Hytten, 1954] – another demonstration that the stroma seems to have nothing to do with the nutritive function of the organ.

One suggestion [Morris, 1967] is that the breast has taken on a secondary role in the human being – that of sexual attraction. The sexual significance of the breast in Western society is beyond question, but it is not clear whether this is merely a cultural peculiarity or whether the role is one for which the breast has been adapted by evolution. Anthropologists report that some primitive peoples regard the breast as a sexual area while others do not [Ford and Beach, 1952]. Since the breast appears to play a sexual role in various cultures – if not universally in our species – there would appear to be some basis for the suggestion that the purpose of the stroma is a sexual one, quite unconnected with the nutritive role of the glandular component.

Among other primates a similar sexual stroma is situated – more logically, perhaps – on the perineum. Remarkable changes in volume and color occur in this area when the female monkey is sexually receptive. Morris [1967] suggests that when the "naked ape" – the human being – assumed its upright posture, changes in the color and volume of the female perineum would be useless as a sexual signal to the male (not to mention being rather inconvenient to the female). The anterior chest wall became the logical area from which the female could signal her sexuality – hence the unique prominence of the nonlactating breast in the human female. This idea might seem fanciful at first sight, but it is well known that the milk-line in all mammals runs from the axilla to the groin, and the particular part that develops into a mammary gland is the area that is most convenient in each particular species. Possibly the stroma of the subdermal layer of the milk-line retains the potential ability to respond to estrogens, and the part that does actually respond is also species-specific.

Whatever the function of the stroma, it is clearly estrogen-sensitive: The first sign of awakening ovarian activity at puberty is the appearance of the breast bud, which usually develops before the appearance of pubic hair and before the menarche [Marshall and Tanner, 1974]. The swelling of the breast at puberty is due mainly to an increase in the stromal component, with only a slight development of the glandular tree [Dawson, 1934]. Since stromal development is often complete, or at least well advanced, by the time ovulation first occurs, it is clear that only estrogens, without the help of progesterone, are necessary for this development.

2. The glandular tissue. It has been pointed out above (Section IA) that the pattern of pregnancy and lactation in the Western culture is an artificial one. The "natural" pattern in primitive cultures – the pattern, it may be argued, for which evolution has prepared our species – is a cycle of pregnancy and lactation, beginning soon after the menarche. Instead of this, women now have a long period of self-imposed infertility after the menarche. During this interval the breast, which has undergone full stromal development but only partial glandular development at puberty, waits for the stimulus of pregnancy to complete the development of the glandular component. This further development in pregnancy is the result of the very marked increase in hormone levels which pregnancy produces: estrogens, progesterone, and prolactin, together with other hormones including insulin, are all involved in producing full lactation. Before the first pregnancy, while the breast is waiting in a condition of partial development, relatively small fluctuations in plasma concentrations of estrogen and progesterone occur as part of the menstrual cycle. After pregnancy and lactation the breast returns to its former state: The glandular tissue regresses to a system of ducts and rudimentary acini, and these structures are again subjected to a series of menstrual cycles before the next pregnancy.

It is easy to imagine the glandular tissue of the breast responding to the smaller cyclical fluctuations in hormone levels with small-scale proliferative changes – the changes of pregnancy in miniature. However, there has been a great deal of controversy over the question of what changes occur in the breast lobules as a result of the menstrual cycle, and indeed over the question of whether changes occur at all. The first investigator to address himself to this question was Rosenburg [1922]: He reported that cyclical changes do occur, but his material was reexamined three years later by Dieckmann [1925], who concluded that the so-called cyclical changes were not a function of the menstrual cycle but a result of the varying ages of the patients. Foote and Stewart [1945] illustrated what they considered to be lobules typical of different phases of the cycle, but they admitted that not all lobules showed these typical changes. Speert [1948] took serial biopsies from the mammary glands of rhesus monkeys, and concluded that in this species cyclical changes in breast lobules do occur: This technique is obviously not appli-

cable to the human – needle biopsy, as used for the detection of malignant cells, does not remove enough tissue for histologic examination.

None of these (and other) investigations on possible cyclical changes took account of the parity of the subjects, or tried to find out whether the breast of the nulliparous woman responds differently during the cycle from the breast which has undergone the full development of pregnancy, lactation, and involution. A research project was therefore undertaken in Edinburgh to answer this question, and the results will be briefly summarized in the next section of this article. This project involved the cooperation of many general surgeons, plastic surgeons, laboratory workers, pathologists, and student volunteers, and it is a pleasure for me to acknowledge their contributions.

II. MENSTRUATION AND THE NORMAL BREAST

A. Gross Anatomic Changes During the Cycle

Many women report a subjective feeling of fullness of the breasts before menstruation, and various attempts have been made to measure the variations in breast size during the cycle. Methods used in the past have included serial X-rays (clearly unethical nowadays), and the making of plaster casts of the breasts – a cumbersome procedure. In a recent study [Milligan et al, 1975] we found that a simple technique of water displacement gave an acceptably low error and a high correlation coefficient between the volumes of right and left breasts. Three nulliparous volunteers measured the volume of each breast daily during a normal cycle, and the results are shown in Figure 1.

It can be seen from Figure 1 that the volume of the breasts increases to a maximum during or just before menstruation. This correlates well with the subjective feeling of fullness that many women experience. The variation in volume is around 20%, and the magnitude of this change shows first that the variation in breast size is much greater than the variation in general body weight, which changes very little during the cycle [Parboosingh et al, 1973], and second that the change must be due to an alteration of some kind in the stroma rather than in the ducts or acini. The glandular component of the breast comprises only between 1% and 10% of the total volume of the resting breast, and does not show a great change in amount during the cycle. The 20% volume change must therefore be due to a change in the stroma – perhaps in its water content or its vascularity: Estrogen causes changes in vascular permeability in the breasts of rabbits [Zeppa, 1969].

Whatever the mechanism of the change, it is clear that the stroma of the breast remains hormone-sensitive. It is worth emphasising, however, that these stromal changes have all been demonstrated only in nulliparous women: No similar study has been conducted with parous subjects, so it has not been definitely proved that the breast which has involuted after pregnancy and lactation still shows these

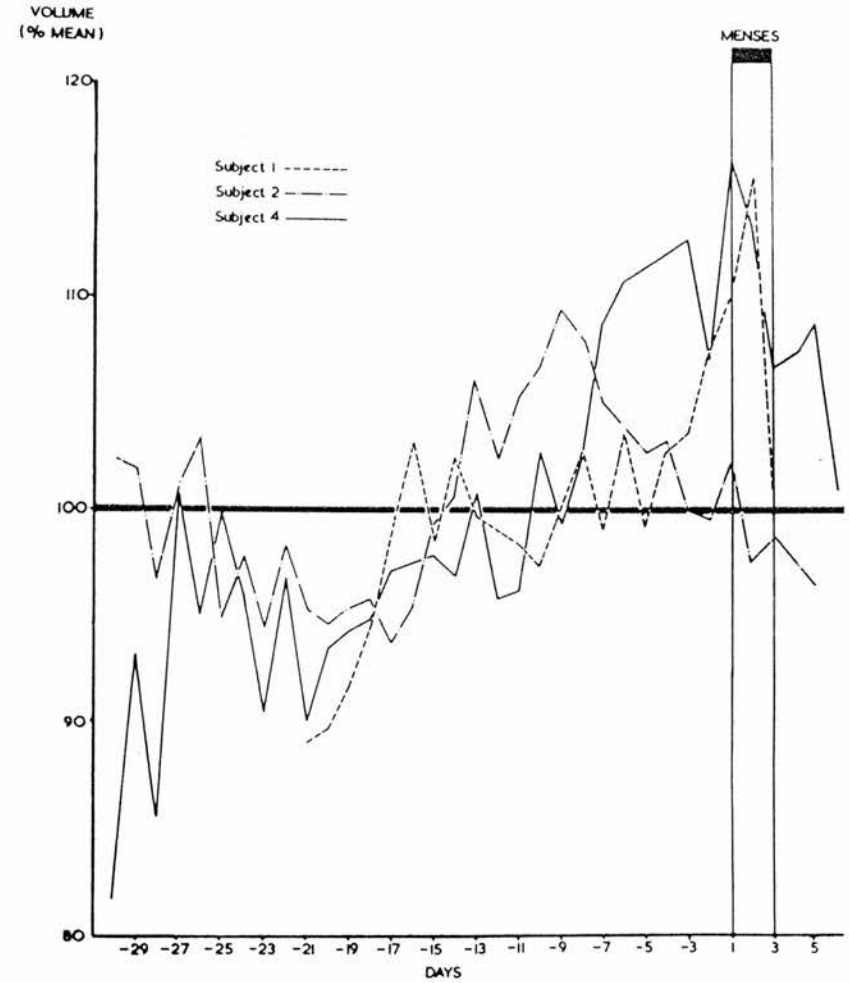

Fig. 1. Breast volume changes during the cycle in three nulliparous women. Volumes are expressed as percentages of the mean volume for each cycle, and plotted backwards from the first day of menses. From Milligan et al [1975], with permission.

stromal changes. However, many women who have borne children still experience a subjective feeling of fullness of the breasts before menstruation, so it seems likely that the stroma remains sensitive to hormones after pregnancy.

B. Histologic Changes During the Cycle

As mentioned above, various investigators have claimed that changes occur in the breast lobules during the cycle. In general, these changes are said to involve increased epithelial growth towards the end of the cycle, along with edema of

the lobules, and increased cellular infiltration of the intralobular stroma. Foote and Stewart [1945] illustrate involution of the lobule beginning after the onset of menstruation – according to these workers, the acini collapse, the cells become pyknotic, and the intralobular stroma becomes dense and infiltrated with lymphocytes. They state that "reactivation" occurs at about day 15 of the cycle, with loosening of the stroma, multiplication of the acini, and secretion into the ductule lumina – changes of such an obvious nature that one wonders why there has been any controversy about them. Because of the fact that parous and nulliparous women were not examined separately, the histologic work was repeated in our project.

Breast biopsies were obtained from 187 women undergoing breast surgery either for cosmetic reasons or for biopsy of a lump. (Patients who subsequently turned out to have breast cancer were excluded from the study.) The tissue for examination was obtained from an area which the surgeon considered macroscopically normal, as far away as possible from the "lump." A full menstrual and reproductive history was taken from each patient and blood was taken for estimation of steroid hormone levels. The biopsies were processed by normal histologic methods and were stained with hematoxylin and eosin.

Estimates were made of the size of lobules, the number of lobules per unit area, the number of acini per lobule, the size of the acini, and the amount of infiltration of the intralobular stroma – factors which previous workers had reported as varying during the cycle. Marked variations were found between specimens taken from different parts of the same breast, and even more variation was found between right and left breasts, where such specimens were available. It proved impossible to confirm the previous claims that lobule size and complexity varied during the cycle, or that the size of the lobules increased steadily during a woman's reproductive lifetime, as some workers have claimed. Lobules "typical" of the various stages of the cycle, as described by earlier investigators, could in fact be found all together in the same breast, and we came to the conclusion that histologic changes during the cycle do not occur, in either parous or nulliparous women. It is of interest that Haagensen [1971] also attempted to confirm the existence of cyclical changes in the breast lobules, and also failed.

The main aim of this histologic study, however, was to identify differences between the breasts of parous and nulliparous women. Despite the difficulty posed by the marked variability in the appearance of the lobules from any one individual, one trend did emerge. Two types of cell have been described in the epithelium of the ductules of the breast – one with pink cytoplasm following staining with hematoxylin and eosin, and one with clear cytoplasm. The pink cells are situated near the lumen of the ductule, while the clear or vacuolated cells are basal in position, and are therefore often thought to be myoepithelial cells [Hamperl, 1970]. However, the fact that the clear cells are sometimes present in larger numbers than other epithelial cells (well illustrated by Haagensen [1971]) casts some doubt on this role [Bassler, 1970]. Whatever the true function of these cells, it has been suggested [Dieckmann, 1925] that vacuolization of the basal layer is characteristic of the premenstrual phase of the cycle – a suggestion that Haagensen [1971] found difficult to confirm.

In our study, epithelia were categorized into three types according to the completeness of the basal layer of vacuolated cells: These three types are illustrated diagrammatically in Figure 2. Estimates were made of the frequency of these types of epithelia in each biopsy, by an observer who was unaware of the histories of the patients from whom they were obtained. It was found that type 1 epithelium was found more frequently among nulliparous women, and type 3 more frequently among parous women – that is to say, the clear or vacuolated cells are more numerous in the breast before the first pregnancy. This trend, illustrated in Figure 3, is statistically significant. Despite Dieckmann's claim [1925] we did not find that the vacuolated cells were more numerous at any particular stage of the cycle.

Although this significant trend was found, it was still no more than a trend, and it was not possible to look at a biopsy and say with certainty that it came from a parous or a nulliparous woman – just as it was impossible to estimate the stage of the cycle at which the biopsy had been taken.

To sum up: The result of this histologic study was to confirm that the breast involutes completely after pregnancy (with or without lactation) and that the lobules do indeed return to their original histologic state. The menstrual cycle does not cause the changes of pregnancy in miniature, in either nulliparous or

Fig. 2. Diagrammatic representation of the three types of epithelium found in the acini on staining with hematoxylin and eosin. In type 1 there is an inner layer of cells with pink-staining cytoplasm and a complete (or almost complete) outer layer of clear or vacuolated cells. In type 2 the two cell types ar intermingled and do not form discrete layers. In type 3, the clear cells are absent.

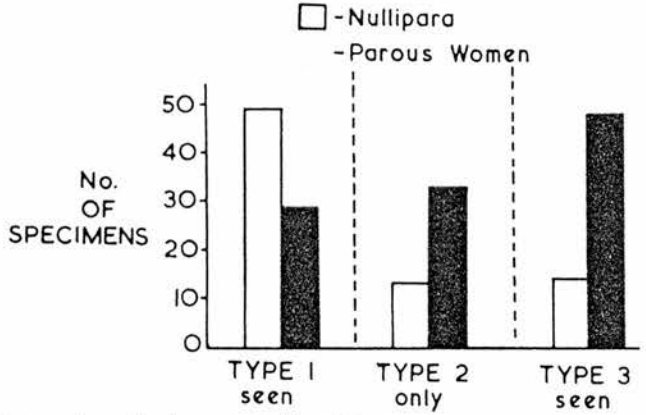

Fig. 3. Histogram shows the frequency with which the three types of epithelium shown in Figure 2 were seen in 187 biopsies of normal breast tissue. Type 1 epithelium was seen mainly in nulliparous women, and type 3 epithelium was slightly commoner in parous women.

parous women. However, there is a suggestion that the epithelium of the ductules is slightly altered by the experience of pregnancy, as shown by the relative numbers of different cell types.

C. Functional Changes During the Cycle

1. Immunoglobulin synthesis. In contrast to the several reports – conflicting though they may be – on the histology of the resting breast, almost nothing is known of its function. The assumption has been that it has none: It has been assumed that glandular activity is absent between puberty and pregnancy, switches on at the end of pregnancy, and switches off again after lactation is over. However, small amounts of secretion can be aspirated from some 75% of resting breasts [Petrakis et al, 1975], and the nature of this secretion is only beginning to be investigated.

Colostrum and milk contain large amounts of immunoglobulin, so we decided to check the immunoglobulin content of our breast biopsies, using an immunofluorescence technique. Also, using a technique of tissue culture with labeled amino acids, followed by radioimmunoelectrophoresis, we tested the ability of 80 freshly obtained breast biopsies to synthesize immunoglobulins [Drife et al, 1976]. We found that 81% of these specimens showed demonstrable synthesis of IgA, while 45% showed IgG synthesis and only 3% IgM synthesis. IgA is the principal immunoglobulin of milk and other glandular secretions, while IgG is found in greater quantities in the blood. The synthesis of IgA was of much greater intensity than that of IgG or IgM.

When the immunoglobulin content of 34 tissue slices was studied by immunofluorescence, much greater quantities of IgA were found than of IgG or IgM: 71%

TABLE I. Influence of Parity and the Menstrual Cycle on In Vitro Synthesis of IgA by Breast Tissue

	Phase of cycle	Intensity of IgA synthesis		
		Strong	Weak	Negative
Nulliparous women	Proliferative	4	7	1
	Luteal	1	5	4
Parous women	Proliferative	3	9	5
	Luteal	16	7	3

Values are the number of specimens showing strong, weak, or no synthesis of IgA. The phase of the cycle was defined by the plasma progesterone concentration: Proliferative, less than 1 ng/ml; luteal, more than 1 ng/ml. Intensity of synthesis was assessed by intensity of labeling of autoradiographs. Modified from Drife et al [1976], with permission.

of specimens had plasma cells containing IgA, and 88% had deposits of IgA within the ductules. Plasma cells containing other immunoglobulins were found in much smaller numbers and were distributed through the stroma of the breast without any concentration in the lobules. Free deposits of IgA were seen most frequently in the lumina of the ductules, less frequently in the stroma of the lobules themselves, and much less frequently in the interlobular stroma: Deposits of the other immunoglobulins were fewer and were more evenly distributed.

It appears, therefore, that a minimal level of immunoglobulin synthesis continues in the so-called resting breast. This was found in both parous and nulliparous women, but when these two groups were looked at separately, it was found that among parous women IgA synthesis was more intense during the luteal phase of the cycle, whereas this effect was not seen among nulliparous women. Table I shows that "strong" IgA synthesis was seen in 16 parous women in the luteal phase of the cycle, but in only one nulliparous woman in the luteal phase – a statistically significant difference. A similar trend was found when the numbers of plasma cells were examined in the immunofluorescence specimens.

Thus the functional ability of the breast seems to have been altered somehow by the experience of one full-term pregnancy. We found no correlation between the ability to synthesize IgA and the total number of pregnancies a woman had had, or the history of lactation, the time elapsed since the menarche, or plasma level of estradiol.

Synthesis of immunoglobulins is carried out by the plasma cells in the stroma of the lobules, and the mechanism of transport of the immunoglobulins into the duct lumen is unknown. Plasma cells are known to migrate into glands from the circulation, but the factor that attracts them into the lobules of the breast is also unknown. It may be a product of the epithelial cells, and so the variation in the

numbers of plasma cells during the cycle in parous women may be the result of variation in the amount of this postulated attractant. However, it is by no means certain that the activity of the plasma cells is an indicator of the functional activity of the glandular epithelium, and even if it is, the indication is only an indirect one. We therefore attempted to investigate the epithelium itself more directly, by studying DNA synthesis in the epithelial cells.

2. DNA synthesis in the epithelial cells. Using a tissue culture technique, the amount of DNA synthesis in the epithelial cells was assessed by incubating fresh tissue slices with labeled thymidine and estimating the incorporation of label by autoradiography. Counts were made of the number of labeled nuclei in the epithelium, and at least 1,000 epithelial nuclei were counted in each specimen [Masters et al, 1977]. The "labeling index" (LI) was expressed as the number of labeled nuclei per 1,000.

There were 52 specimens examined from 47 patients, and duplicate specimens from the same breast or from both breasts confirmed the repeatability of the results. We found that DNA synthesis, like immunoglobulin synthesis, showed a cyclical pattern only among parous women. The decreasing activity during the follicular phase and the sharp increase during the luteal phase of the cycle are shown in Figure 4. By contrast, no cyclical activity was seen in the specimens

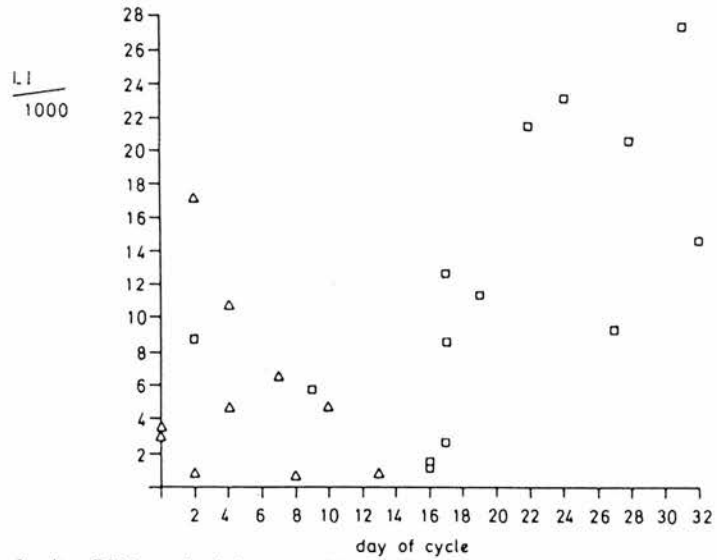

Fig. 4. In vitro DNA synthesis by normal breast tissue from parous women: Relationship between epithelial cell lableing indices and day of the cycle. Triangles denote specimens from women with a plasma progesterone level less than 1 ng/ml; squares denote those where the level was over 1 ng/ml, signifying the presence of a corpus luteum. From Masters et al [1977], with permission.

from nulliparous women (Fig. 5). No relationship was found between LI and time since menarche, breast feeding, or plasma concentration of estradiol.

The exact significance of the change in labeling indices is not clear: Increased LI indicates an increased cell growth rate, but conclusions cannot be drawn about the exact rate of growth. We conclude that among parous women an increased rate of cell growth does occur in the second half of the cycle, but that this cell growth does not go so far as to produce detectable changes on histologic examination.

D. Differences Between Nulliparous and Parous Women

Over the series of investigations summarized above, a trend has emerged. In the breast of the nulliparous woman, only the stroma is sensitive to hormone fluctuations of the menstrual cycle, and the glandular epithelium itself shows no discernible signs of histologic or functional change during the cycle. In parous women, on the other hand, the glandular component does show variations during the cycle, and the stroma may also be sensitive to hormone fluctuations (though this has not been investigated). Though histologic changes do not occur during the cycle in parous women, there are functional changes, with increased activity during

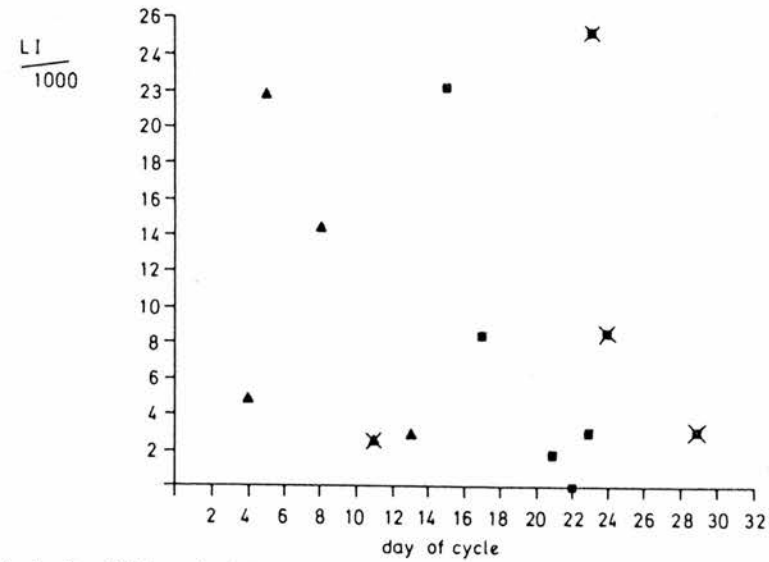

Fig. 5. In vitro DNA synthesis by normal breast tissue from nulliparous women: Relationship between epithelial cell labeling indices and day of the cycle. Triangles denote specimens from women with a plasma progesterone level less than 1 ng/ml; squares denote those where the level was over 1 ng/ml. Crosses denote specimens obtained at reduction mammoplasty. From Masters et al [1977], with permission.

the luteal phase. There is evidence of a slight histoligic difference between the breasts of nulliparous and parous women.

I suggest that the breast of the nulliparous woman should be regarded as a "immature" organ, and that only after the first full-term pregnancy does the breast reach its final mature stage. During the immature stage between menarche and first pregnancy the epithelium is only partially sensitive to steroid hormones: The cells require the stimulus of the relatively high steroid levels in pregnancy before they can reach their full functional maturity, with the ability to respond to steroid hormones. By the end of pregnancy profound structural and functional changes have been produced in the breast, and after the breast has reached this stage – whether lactation is suppressed or not – it never reverts completely to its immature state.

It has already been pointed out (Section I) that until this century the immature stage of development was a relatively short one. In the circumstances under which the breast originally evolved, its development from the first budding at puberty to full lactation would have been a relatively smooth process, beginning before the menarche – which would be followed quickly by the first pregnancy. It is only nowadays that we have begun to hold the breast in the partially developed stage for a decade or more. The possible harmful effects of this will be further considered in Section IV, but the next section will take up another very recent influence that affects the breast – the use of the oral contraceptive pill.

E. Effects of Oral Contraceptives

Oral contraceptives are now widely used throughout the world, by both nulliparous and parous women. They are rather more suitable for the younger age groups, and tend to be the contraceptive method of choice for a woman before her first pregnancy. A careful watch has been, and is being, kept on their possible ability to cause breast disease, but otherwise their effect on the resting breast has hardly been examined. Therefore, in each of the investigations mentioned above, we also included women taking oral contraceptives.

In the study of breast volume changes, three volunteers measured the change in breast volume during courses of the combined oral contraceptive pill. The results are shown in Figure 6. The pattern of volume change was broadly similar to that during the normal cycle, with a steady increase during the 21 days of treatment and a quick decrease during the week "off" the Pill. The amount of change was again of the order of 20%. What happens if a woman continues to take the Pill without a break? The volume increase does not continue indefinitely: In one trial in which oral contraceptives were taken for three months continuously [Loudon et al, 1977], 13% of the subjects complained of breast discomfort, which became less troublesome as the trial progressed. It would appear, therefore, that the stroma has only a limited ability to respond to continuous exposure to steroids.

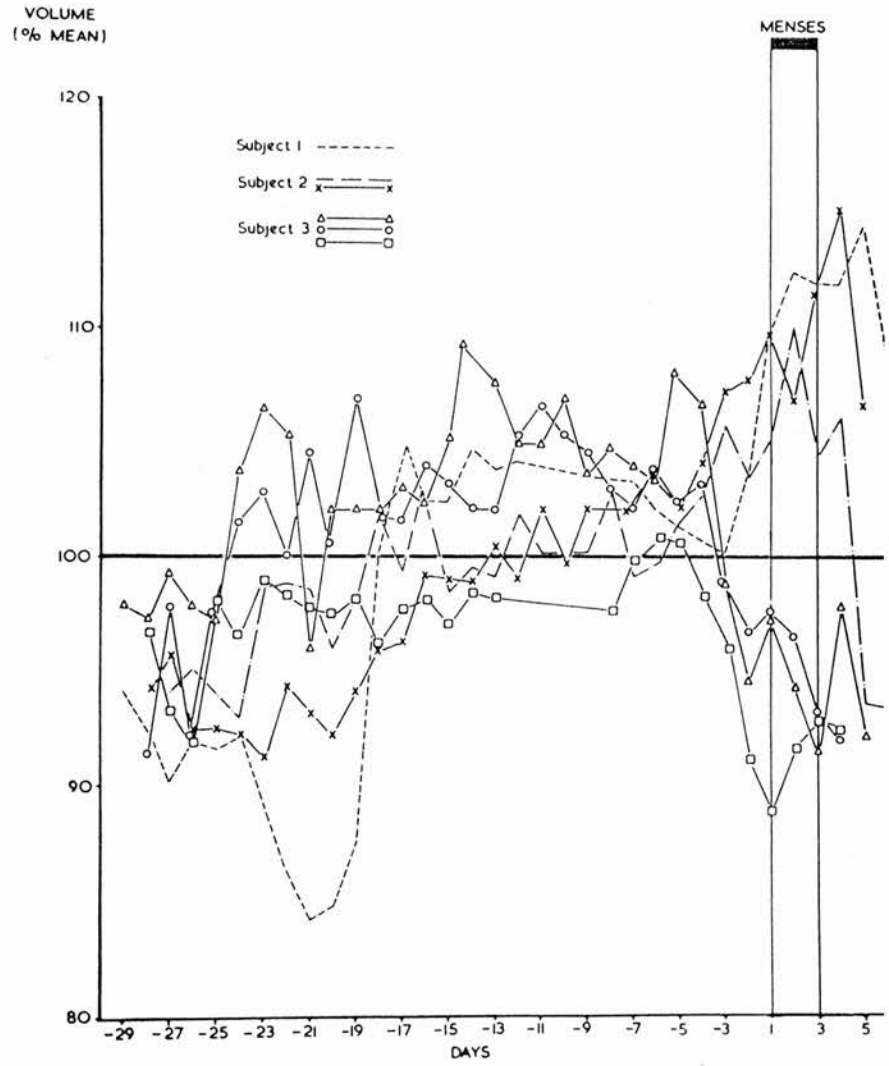

Fig. 6. Breast volume changes throughout six complete contraceptive-controlled cycles in three nulliparous women. Volumes are expressed as percentages of the mean volume for each cycle, and plotted backwards from the first day of menses. From Milligan et al [1975], with permission.

When the histology of the breast was examined, no effect of oral contraceptives could be demonstrated. Separate examinations were made of material from nulliparous and parous women, and counts were made of lobules, lobule area, acini per lobule, size of acini, and cellular infiltration: There was no indication in either

group of a difference between women on the Pill and normal subjects. There was no evidence that the use of the Pill either stimulated or suppressed the glandular component of the breast.

In our tissue culture investigations, patients on the Pill were also examined, and the results for both immunoglobulin synthesis and DNA synthesis were scattered within the normal range found in subjects who were not on the Pill. There was no sign that cyclical changes occurred during the month on the Pill in either nulliparous or parous women.

The effects of the Pill on the resting breast appear, therefore, to be limited to two areas. First there is a stimulation of the stroma, analogous to the change seen in the normal cycle, and second there is an abolition of the normal cyclical response of the glandular component of the breast of the parous woman. Could either or these effects be harmful – or even beneficial? Neither possibility seems likely at the moment, in view of the fact that the incidence of breast disease is hardly altered by the Pill [Vessey, 1978], but the effects of oral contraceptives on breast disease will be considered below.

III. EPIDEMIOLOGY OF BREAST CANCER

A. Hazard of Prolonged Nulliparity

It is well known that breast cancer is commoner among nulliparous women, but the reason remains obscure. Much investigation has been carried out by epidemiologists to clarify the exact nature of the connection between parity and cancer risk, but it remained controversial until the large surveys of MacMahon et al [1973] and Shapiro et al [1973]. Both of these surveys examined a wide range of possible risk factors: Some proved to have no connection with breast cancer, while some -- such as age at the menarche, or early oophorectomy – were indeed shown to be related to breast cancer risk. One of the more striking findings was the relationship between the risk of subsequent breast cancer and the age at which a woman has her first full-term pregnancy. This is illustrated in Figure 7, the graph that was first published by MacMahon et al in 1970.

The linear relationship between breast cancer risk and age at first birth, shown in Figure 7, holds for different races and is unaffected by the number of subsequent pregnancies. It is also clear from the same survey (as well as from other work) that an early menarche increases the risk of breast cancer later in life [MacMahon and Cole, 1972]. Therefore, although other risk factors, such as race, are also important, one significant factor in determining the risk of later breast cancer is the length of time that the breast remains in its immature state before the first full-term pregnancy. Once the massive hormone stimulus of pregnancy brings about full development of the glandular component this particular risk is removed, no matter how long the breast remains in its resting state after maturity has been reached.

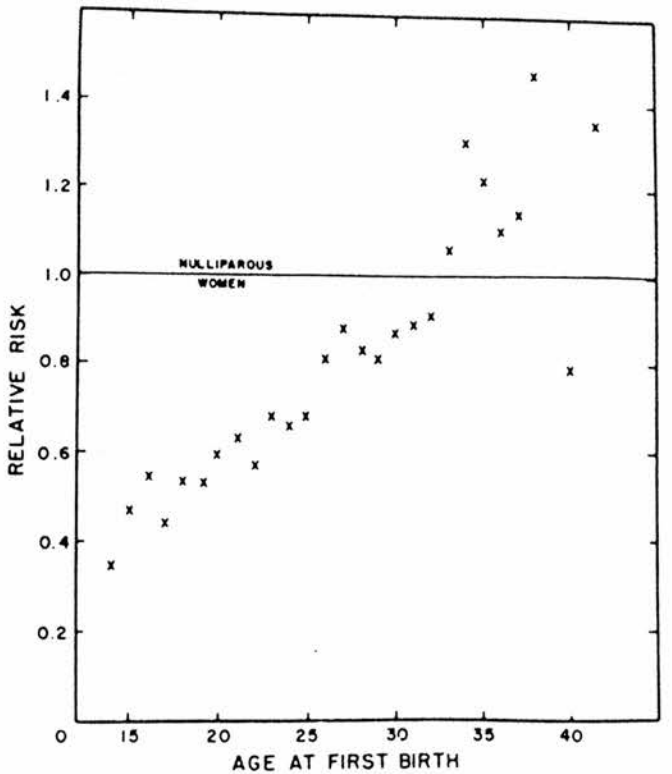

Fig. 7. Relative risk of developing breast cancer later in life, in relation to a woman's age at the birth of her first child. The risk in nulliparous women is taken as 1.0. From MacMahon et al [1970], with permission.

Epidemiologic evidence has not indicated the nature of the carcinogenic stimulus during the years before the first full-term pregnancy. Perhaps the immaturity of the gland itself, without any other outside influence, is enough to cause instability of the epithelial cells, leading to cancerous change -- in the same way that the incompletely developed undescended testis is liable to malignant change. However, the significance of the age at menarche makes it evident that the precancerous process begins at puberty, not at birth, and this observation has raised the suspicion that ovarian hormone production is implicated in the precancerous process. Estrogens have often been cast in the role of carcinogens [MacMahon and Cole, 1972], and they certainly appear to be prime suspects, since puberty seems to mark the start of exposure to a carcinogen which continues to act until the gland reaches maturity.

An alternative explanation for the significance of the age at menarche is that puberty itself produces a change in the cells, after which their intrinsic immaturity

makes them more unstable and susceptible to precancerous change. However, puberty does not involve a sudden surge of hormone production but a gradual buildup of estrogen concentrations until the mature levels of the reproductive years are reached. It seems unlikely that this slow buildup would produce a dramatic once-and-for-all change in the glandular cells. We are left with the strong possibility that exposure to estrogen is indeed the carcinogenic influence.

B. Previous Theories of Hormonal Causation of Breast Cancer

A connection between estrogens and breast cancer has long been suspected for a number of reasons, including the fact that early oophorectomy protects against the disease. The mechanism by which the ovary acts on the breast has remained obscure, however, and the possibility has been raised that different types of estrogen may have differing potentials for carcinogenesis [MacMahon and Cole, 1972; Kirschner, 1977].

It was found that patients with breast cancer excrete relatively less estriol than estradiol or estrone, and since estriol is a less potent estrogen in bioassay systems than the other two, it was suggested that it might protect the breast from the higher carcinogenicity of the two more potent estrogens. Excretion of estriol among groups of women of varying nationalities at varying risks of breast cancer was found to vary inversely with the national incidence of the disease. However, this "estriol hypothesis" is not universally accepted, partly because it is not in fact certain that estriol is less carcinogenic than the other estrogens (see Kirschner [1977] for review). Siiteri et al [1974] have presented reasons for suspecting that the carcinogen is estrone rather than estradiol, and they have formulated an alternative "estrone hypothesis."

There has been less interest in the role of progesterone in the development of breast cancer, although progesterone normally acts on estrogen-primed target tissues. Grattarola [1964] found that breast cancer patients had a higher incidence of anovulatory cycles than normal women, and Sherman and Korenman [1974] suggested that inadequate corpus luteum function may be very common among breast cancer patients. These observations point to the possibility that progesterone has some protective effect on the breast – possibly counteracting to some extent the carcinogenic effect of estrogen. If the protective effect of progesterone is deficient, the chances of breast cancer developing are higher.

Such a protective role for progesterone would be in keeping with observations on endometrial cancer, which is commoner among patients with excess estrogen production (eg, by granulosa cell tumors of the ovary) and among patients in whom the effect of estrogen is unopposed by progesterone: Under such circumstances estrogen produces hyperplasia of the endometrium, which may be followed by frank cancer. The possibility has also been raised that unopposed exogenous estrogens, taken over a period of years, may have a carcinogenic effect on the endometrium [Ziel and Finkle, 1975]. It is now believed that the possible carcinogenic effect of exogenous estrogens on the endometrium can be modified by the addition of progesterone to estrogen therapy [Studd et al, 1978]. Hoover et al [1976] have suggested that a similar carcinogenic effect of unopposed exogenous estrogens might exist for breast cancer, but the possibility of abolishing this effect by the addition of progesterone to estrogen therapy remains to be investigated.

C. Effects of Oral Contraceptives

When oral contraceptives were first introduced there were fears that continued use of potent steroids might increase the incidence of breast cancer. Others hoped that the suppression of endogenous hormone production might actually decrease the incidence of the disease. Studies so far have not justified either the hopes or the fears [Vessey, 1978; Startwell et al, 1977]. It has been tentatively suggested that use of the Pill before first childbirth may be associated with an incrased risk of breast cancer [Pfaffenbarger et al, 1977], but such an increase (if it exists at all) must be only slight and may in fact be due to the delaying of the woman's first pregnancy. On the other hand, the hope that the Pill may decrease the incidence of breast cancer has been kept alive by the finding that benign breast disease (which has a limited association with breast cancer) is rather less common among women on the Pill.

It is, of course, still too early to assess with certainty the effect of oral contraceptives on breast cancer, since the first users of the Pill are only now beginning to reach the age at which they are at greatest risk of breast cancer. However, the indications at present are that there will not be a dramatic change in incidence attributable to the Pill.

IV. MENSTRUATION AND BREAST CANCER

A. The Nature of the Connection

The results presented in Section II point to the conclusion that the menstrual cycle has different effects on the breasts of parous and nulliparous women. Among parous women, an increase in the activity of the glandular tissue during the second half of the cycle reflects the stimulation of the breast by progesterone from the corpus luteum. Among nulliparous women, this increase is absent. The plasma concentrations of progesterone in the luteal phase (and of estradiol) were found in our study to be no different in nulliparous and parous women, so the conclusion is that the glandular tissue of the breast is not responsive to nonpregnant levels of progesterone until after the first pregnancy. This means that before the first pregnancy the estrogen present for most of the cycle is effectively "unopposed" by progesterone, despite the latter hormone's presence in the blood in adequate concentration. Once the hormonal stimulus of the first pregnancy has brought the

glandular tissue to full maturity, circulating progesterone can act on the epithelial cells to oppose estrogen. If estrogens are indeed carcinogenic, as is now widely believed, this phenomenon provides a satisfactory explanation of the precancerous stimulus acting between menarche and first pregnancy.

The observation that the stroma of the breast is also hormone-sensitive may be of some relevance to this hypothesis. If there are estrogen receptors in the breast stroma they will bind the hormone, removing it from the circulation and retaining it in higher concentration near the glandular epithelium.

This "progesterone block" hypothesis extends but does not challenge the theories of hormonal carcinogenic action referred to above (Section III). Sherman and Korenman's suggestion [1974] that inadequate luteal phases predispose to breast cancer is not challenged: If inadequate luteal phases occur *after* the first pregnancy, estrogen action will of course be unopposed by progesterone at that time, in spite of the fact that the breast tissue is by then capable of responding to progesterone when present. It is even possible that inadequate luteal phases before the first pregnancy may have an effect – if the immature breast's insensitivity to progesterone is not complete but only partial. However, our results suggest that the block to the action of progesterone on the immature breast is complete, since cyclical activity was completely absent in nulliparous women, and therefore I suggest that it is immaterial whether the luteal phase *before* first pregnancy is adequate or not.

This hypothesis does not directly challenge the estriol or the estrone hypotheses. The nature of the circulating estrogen – whether it is predominantly estrone, estradiol, or estriol – and the relative carcinogenicity of each, are irrelevant to the hypothesis that they are all acting without the opposing or modulating effect of progesterone.

Finally, this hypothesis fits the observation that the Pill has not changed the incidence of breast cancer. When a woman takes the Pill her endogenous estrogen product is suppressed by the exogenous estrogen – there is therefore still circulating estrogen acting on the breast. There is, of course, also a progestogen in the combined Pill, but if the woman is nulliparous, her breast will be insensitive to this, and the end result, as far as the precancerous process is concerned, will be unchanged.

B. Clinical Relevance

The hypothesis outlined above might at first sight appear to offer little hope of finding a way to decrease the incidence of breast cancer. Nothing can be done at present to change the properties of the epithelial cells themselves and make them more responsive to progesterone. The age at menarche is not under our control. There is unlikely to be a reversal of the social trend towards delaying first pregnancy until a woman has established a career and a home. If the precancerous process affecting the breast between menarche and first pregnancy is in fact the result of an innate instability of the cells themselves, there is indeed little that we can do in the present state of our knowledge.

If, however, the precancerous process is the result of unopposed estrogen action on the breast epithelium, as suggested here, this opens the possibility of reducing the incidence of breast cancer by completely suppressing ovarian activity before first pregnancy – without using exogenous estrogen. None of the contraceptive methods available at the moment removes estrogen from the circulation. Baird [1976] has suggested that it should be possible to develop a new kind of contraceptive which suppresses the hypothalamic-pituitary-ovarian axis without providing exogenous estrogen. For example, before puberty endogenous estrogen production is minimal because of the very high sensitivity of the hypothalamus to the minimal levels of circulating estrogen. At puberty this hypothalamic sensitivity decreases – a change that is very poorly understood at present. Future research in contraception could be directed towards developing ways of mimicking such physiologic amenorrhea. Only further work would show whether or not such amenorrhea would be acceptable to the majority of women, but already a trial has demonstrated that three-month intervals of amenorrhea are acceptable to most women. [Loudon et al, 1977].

It is worth pointing out also that a new form of contraception which reduced the number of menstrual periods that a woman has to endure would have other advantages besides reducing the action of estrogen on the breast. Monthly hemorrhages cause women pain, anemia, depression, and other unpleasant physical and psychologic effects. The monthly withdrawal bleeds of women on the oral contraceptive pill are unnecessary, except to reassure a woman that she is not pregnant, and they are the cause of many Pill "failures" – since follicles begin to mature during the week "off" the Pill, and can release ova if further stimulated by the woman forgetting to take her Pill correctly later in the month. Like many other areas of medical and scientific endeavor, the development of contraception so far has been male-dominated: Women scientists might perhaps have been more ready to question whether monthly bleeding is necessary at all.

The hypothesis outlined in this article also highlights the importance of gaining more knowledge about the action of hormones on the normal breast – a sadly neglected field. Since the search for improvements in results from our treatment of breast cancer is proving so disappointing, it would seem logical to direct more attention towards ways of avoiding the disease. The importance of steroid hormones in the causation of breast cancer is obvious, and their influence is one of the few risk factors that should be susceptible of modification. Now that the epidemiology of breast cancer is better understood, more resources ought to be directed towards study of the organ in which the precancerous change begins – the normal breast.

ACKNOWLEDGMENTS

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clic Variation of DNA Synthesis in Human Breast Epithelium[1]

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STRACT—DNA synthesis in normal breast epithelium from menopausal women was assessed by use of autoradiogra-. In parous women the labeling indexes decreased during the cular phase of the menstrual cycle and increased to a signifi-tly higher level during the luteal phase.—J Natl Cancer Inst 1263-1265, 1977.

he risk of breast cancer is three times greater among men whose first full-term pregnancy is delayed until 35 or over than among those who bear their first ld before the age of 19 (*1*). A possible explanation for observation is that the breast epithelium responds to hormone fluctuations of the menstrual cycle and this response is altered following first pregnancy. support of this hypothesis is the report that the ability breast tissue to synthesize immunoglobulins is differ- in parous and nulliparous women (*2*). This is the t study to report cyclic variation of DNA synthesis in ast epithelium during the menstrual cycle.

TERIALS AND METHODS

he specimens of normal breast tissue were obtained m reduction mammoplasties or at biopsy from an a regarded by the surgeon as normal tissue, follow- removal of abnormal tissue. In all cases the breast ue was histologically confirmed as normal. The 52 cimens were obtained from 47 patients; a further 21 cimens were discarded because insufficient epithelial s were present. The nature of the abnormal area, ermined by routine histopathology, was in all cases ign.

he specimens, approximately 5×3×2 mm, were im-diately transferred to Waymouth's MB752/1 medium taining 20 mM HEPES[7] (Flow Laboratories, Irvine, tland). Within half an hour of surgery the tissue was into 1-mm slices with paired razor blades and trans-ed to 5 ml of fresh medium (pH 7.2) containing 2 [*methyl*-^{3}H]thymidine/ml (Radiochemical Centre, ersham, Buckinghamshire, England; 5 Ci/mmole) in aled plastic Universal container. After 4 hours' incu-on at 37° C, the tissue was washed three times in nks' balanced salt solution and fixed in formol saline. he tissue was processed by routine histologic proce-es, and 5-μ paraffin sections were picked up on ted slides. Autoradiographs were prepared with Ko- AR10 stripping film and exposed for 14 days at . They were placed for 5 minutes in Kodak D19 eloper and fixed for 10 minutes in Ilford Hy-n:distilled water (1:5); both processes were carried at 17° C. The sections were washed, dried, stained n hematoxylin and eosin, and mounted in DePex.

t least 1,000 epithelial cell nuclei were counted in n specimen (average count: 1,446), and the number abeled nuclei were noted; any nucleus with more n 5 grains was scored as positive. The LI was ex-ssed as the number of labeled nuclei per 1,000 after 4 hours' incubation with tritiated thymidine. The results were statistically analyzed by the Wilcoxon rank sum test.

A 10-ml venous blood sample was collected from each patient within 24 hours before or at the time of operation, and the plasma was stored at −20° C. The concentrations of progesterone (ng/ml) and 17β-estradiol (pg/liter) were determined by radioimmunoassay (*3, 4*). Women were classified as being in the luteal phase if the progesterone concentration exceeded 1 ng/ml. Details of parity, first day of last menstrual period, and number of years since menarche were obtained from each patient.

The repeatability of the results was tested with tissues from 4 patients. The results were: 1) right and left breasts, LI: 3.0 and 1.9; 2) right and left breasts, LI: 3.0 and 1.8; 3) two specimens from the same breast, LI: 20.0 and 22.2; and 4) three specimens from the same breast, LI: 4.2, 3.7, and 4.6. These results indicated that different specimens obtained from the same or contralateral breast showed comparable LI.

RESULTS

The results are summarized in table 1. In parous women the LI of the breast epithelium decreased during the follicular phase and then increased to a significantly higher level ($P<0.05$) during the luteal phase of the menstrual cycle (text-fig. 1). No significant difference was observed in the menstrual ages (yr since menarche) between these two groups. In the nulliparous group cyclic variation was not demonstrated, although the number of specimens in the group was small (text-fig. 2).

No cyclic variation was observed in the LI of breast epithelium from women using oral contraceptives, and the LI were uniformly low (table 1). The LI of the parous group using oral contraceptives were similar to those of the parous follicular group, although no signifi-

ABBREVIATION USED: LI = labeling index(es).

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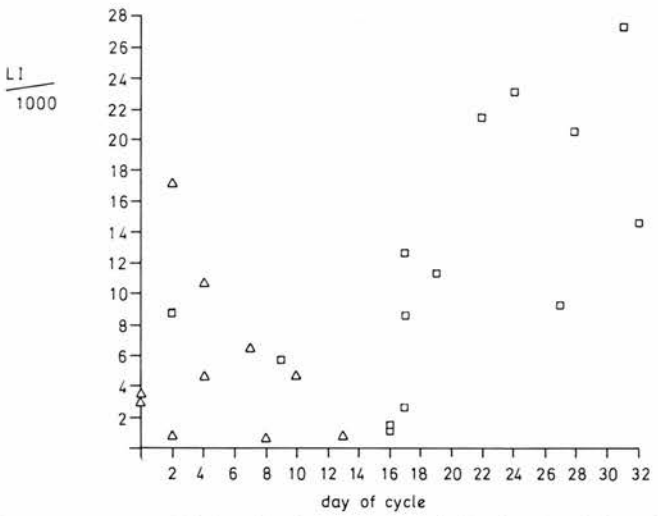
[7] HEPES=*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

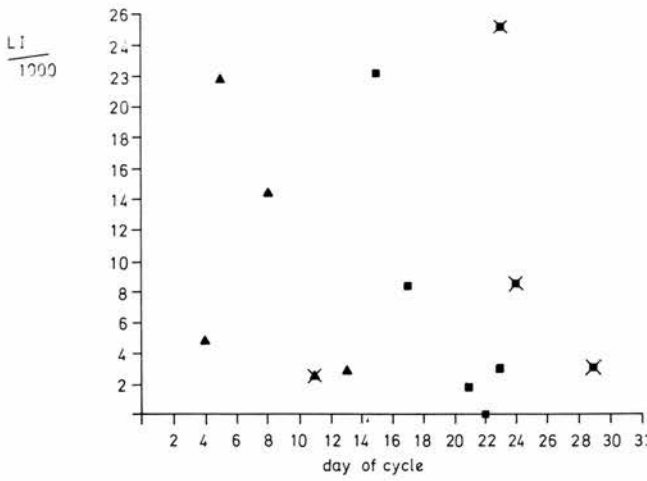
TABLE 1.—*Epithelial cell LI of normal breast tissues and the menstrual ages of 47 women*[a]

Normal breast tissue from patients undergoing surgery for:	Parity	Phase of menstrual cycle	Number of patients	LI/1,000	Menstrual age
Fibroadenoma or mammary dysplasia	Parous	Follicular	10	0.8–17.1	14–33
		Luteal	14	1.6–27.4	11–32
		Oral contraceptive	7	2.0–7.0	4–25
	Nulliparous	Follicular	4	3.0	4
				4.8	6
				14.6	5
				21.8	3
		Luteal	5	0.0	6
				1.9	30
				2.9	10
				8.3	8
				22.0	35
		Oral contraceptive	2	1.3	7.5
				8.3	10
Reduction mammoplasty	Nulliparous	Follicular	1	2.5	9
		Luteal	3	1.9; 3.0	8
				8.5	5
				25.2	6
		Oral contraceptive	1	4.2; 3.7; 4.6	15

[a] Patients were divided by reason for operation, parity, and phase of menstrual cycle.



TEXT-FIGURE 1.—Relationship between epithelial cell LI and day of cycle in specimens of normal breast tissues from parous women in the follicular (△) and luteal (□) phases of the menstrual cycle. The specimens were obtained from premenopausal women undergoing surgery for benign breast lumps.



TEXT-FIGURE 2.—Relationship between the epithelial cell LI and of cycle in specimens of normal breast tissues from nullipar women in the follicular (▲) and luteal (■) phases of the menstr cycle. The specimens were obtained from premenopausal wor undergoing surgery for benign breast lumps or reduction mam plasty (X).

cant difference was seen between the LI of the former group and those of the parous luteal group.

The LI were compared with the plasma 17β-estradiol concentrations and the menstrual age, but no associations were apparent.

DISCUSSION

Changes in total breast volume (*5, 6*), amount of intralobular stroma (*7, 8*), and the size of the alveoli have been reported to occur during the menstrual cycle. This study has investigated the level of DNA synthesis in normal human breast epithelium; although specific conclusions regarding cell growth rates cannot be made from LI alone, alterations in the number of labeled cells do indicate a change in the pattern of growth. In parous women the LI of the breast epithelium decreased du ing the follicular phase and increased during the lut phase of the menstrual cycle. Increased labeling duri the luteal phase concurs with previous histologic obs vations (*7*), which suggest greater activity during t phase. Material was insufficient to determine whet similar cyclic changes occur in the breast epithelium nulliparous women; therefore, further investigation w be necessary to determine whether differences in t growth rates of breast epithelium exist between paro and nulliparous women. Nevertheless, the pres study is the first to demonstrate quantitative changes the behavior of human breast epithelium during t menstrual cycle. This finding is particularly signific because the epithelial cells are the most common site malignant change.

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Immunoglobulin synthesis in the "resting" breast

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Summary

Nulliparous women have a greater risk of developing breast cancer than women who have borne children, but so far no functional differences in breast tissue have been reported between parous women and nulliparae. Macroscopically and histologically normal breast tissue was obtained from 74 women of reproductive age during biopsy of benign breast lesions and was examined for the presence of plasma cells by immunofluorescence. Immunoglobulin synthesis was detected by an in-vitro culture technique.

Synthesis of IgA was detected in 81% of specimens, of IgG in 45%, and of IgM in 3%. IgA synthesis was much more intense than IgG or IgM synthesis. Plasma cells containing IgA were seen in 71% of the specimens examined, and 88% of specimens had deposits of IgA in the ductules. The findings were not significantly influenced by the nature of the condition necessitating biopsy or by oral contraception. Nulliparous women showed no cyclical changes, but among parous women IgA synthesis was more intense during the luteal phase of the menstrual cycle. This suggests that after the first pregnancy the breast is more sensitive to progesterone.

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Introduction

The term "resting breast" is often applied to the human mammary gland that is not secreting milk, implying that the gland is metabolically inactive. Small amounts of secretion can be aspirated from at least 75% of non-lactating breasts,[1] however, and changes in breast volume during the menstrual cycle suggest that the non-lactating breast responds to fluctuating levels of ovarian hormones.[2]

Although milk-specific proteins and large amounts of immunoglobulin[3] are secreted in milk, there is little information about the ability of the non-lactating breast to produce these proteins. The purpose of our study, which was part of a wider investigation of the effects of parity and the menstrual cycle on the breast, was to determine whether the resting breast produces a milk protein (IgA) and whether such synthesis varies with the stage of the menstrual cycle.

We therefore studied immunoglobulin production in the non-lactating human breast using an in-vitro organ culture technique and also examined the distribution of immunoglobulins by immunofluorescence.

Patients and methods

We studied 74 women of reproductive age (mean age 33 years; range 15-52) with regular menstrual cycles. They had been admitted to hospital for diagnostic biopsy of a breast lump or for reduction mammoplasty. Before surgery each patient was invited to participate in the study, and a full reproductive and menstrual history was taken. To ascertain the time of ovulation venous blood was taken at the time of surgery for progesterone assay, and the patient was asked to notify us of her next menstrual period. Patients whose biopsies showed malignant disease of the breast were excluded from the study.

BIOPSY TECHNIQUE

After the clinically abnormal lump was removed a piece of macroscopically normal breast tissue was removed through the same incision from a site as far away as possible from the abnormal area. Reduction mammoplasty specimens were randomly selected from the excised tissue. The tissues were transported in normal saline and prepared for culture and histological examination within one and a half hours. Adjacent blocks were fixed in 4% neutral buffered formaldehyde.

IMMUNOLOGICAL METHODS

In-vitro synthesis of immunoglobulins—Eighty specimens, each weighing 100-150 mg, were cultured for 48 hours in medium containing ^{14}C-labelled lysine and isoleucine.[4] The cultures were tested for the presence of newly synthesised immunoglobulins and secretory component by radioimmunoelectrophoresis as described.[5] The degree

of immunoglobulin synthesis was assessed semi-quantitatively by grading the intensity of the autoradiographic image from ± (line barely visible) to +++ (a strong black line on the autoradiograph) (see fig 1). The grading was done independently by two observers.

Immunofluorescent detection of immunoglobulin in tissues—Tissue blocks were processed by the method of Brandtzaeg,[6] fixed in ethanol at 4°C, and embedded in paraffin. Sections were stained by direct immunofluorescence using fluorescein-conjugated rabbit antisera against human α-, γ-, and μ-chains (Hoechst Pharmaceuticals), whose specificity has been described.[7] Control experiments were performed by blocking with unconjugated antisera and by using absorbed conjugated antisera. The sections were examined by incident ultraviolet illumination. Sections stained with fluorescent antibody to IgA, IgG, and IgM were graded from ± to +++ both for the number of fluorescent plasma cells and for the number and intensity of fluorescence of non-cellular deposits. A separate assessment was made of deposits within the lumina of ductules, deposits in the intralobular stroma, and those in the extralobular stroma.

HISTOLOGY OF TISSUE STUDIED

Sections cut serially with those used for immunofluorescence were stained with haematoxylin and eosin and examined by two observers, including a pathologist who had no knowledge of the patients. Any specimens in which pathological lesions were found were excluded from the study. Sections from the adjacent formalin-fixed blocks were also examined, and no frank pathological features were seen, though a few showed minor changes (atrophic changes in five specimens, small cysts in two, ductular dilatation in seven, and features of fibrosing adenosis in three). No specimens showed epitheliosis.

HISTOPATHOLOGY OF PRIMARY CONDITION

Patients included in the study had either benign breast lesions (biopsy cases) or no disease (mammoplasty cases). The histological findings on the clinically abnormal tissue are shown in table I. Group 1 consisted of patients in whom the disease would be expected to be confined to the excised lump—for example, fibroadenoma—with the remainder of the breast consisting of normal tissue. In group 2 the diagnosis was fibrosing adenosis, which may not necessarily have been localised to the lump excised, even though the tissue used in our study was histologically normal.

TABLE I—*Histopathological diagnosis of breast lesions in patients from whom normal breast tissue was obtained for in-vitro culture*

	Diagnosis	No of specimens for in-vitro culture	No of specimens also studied by immunofluorescence
Group 1	Fibroadenoma*	20	8
	Mammoplasty*	10	4
	No abnormal tissue	7	3
	Lipoma	1	1
Group 2	Fibrosing adenosis with or without cyst formation*	42	18
	Total	80	34

*Some patients in these groups provided more than one specimen: 74 patients provided specimens for culture and 31 for immunofluorescence.

STEROID ASSAYS

Plasma progesterone levels were determined by a modification of the radioimmunoassay developed by Neal *et al*,[8] and oestradiol-17β levels by a modification of the radioimmunoassay of Cameron *et al*.[9]

Results

IN-VITRO SYNTHESIS OF IMMUNOGLOBULINS

Synthesis of IgA was detected in 81% of the 80 specimens (fig 1) and synthesis of IgG in 45% (table II). IgA synthesis was strong (++ or +++) in 42% of the positive specimens, whereas 94% of the IgG-positive specimens showed only weak or barely detectable synthesis (+ or ±). IgM synthesis was seen in two specimens and was barely detectable in both.

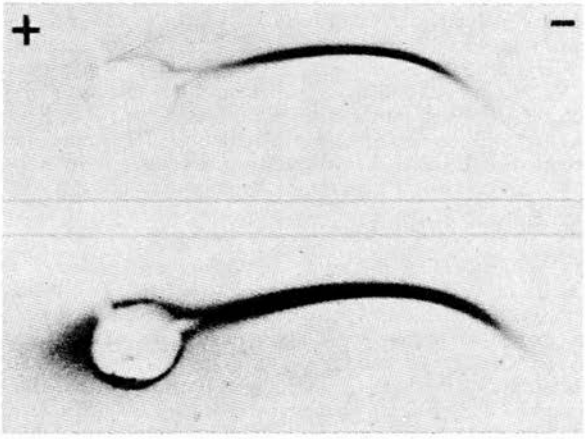

FIG 1—Immunoglobulin synthesis in vitro detected by radio-immunoelectrophoresis. Above: stained immunoelectrophoresis pattern developed with anti-IgA serum showing IgA arc. Carrier is human milk. Below: autoradiograph showing labelling of IgA arc.

Influence of diagnosis—The number of positive specimens and the pattern of synthesis was similar in groups 1 and 2. The proportion of positive specimens was greater in group 2 (see table II), but the difference was not significant by the χ^2 test ($0{\cdot}5 > P > 0{\cdot}1$).

Influence of parity and stage of menstrual cycle—Similar comparisons were made between the parous and nulliparous patients, and the proportions of positive specimens (81% and 77% respectively) were not significantly different. There was no significant difference between tissue removed during the luteal phase of the cycle (as shown by a plasma progesterone concentration greater than 3·2 nmol/l (1 ng/ml)),

TABLE II—*Synthesis of immunoglobulins by breast tissue cultured in vitro*

	No of positive specimens (and % of total)	Mean intensity of labelling of autoradiographs (No of specimens)			
		+ + +	+ +	+	±
	All specimens (n = 80)				
IgA	65 (81)	9	18	24	14
IgG	36 (45)		2	14	20
IgM	2 (3)				2
	Histologically normal tissue from group 1 (n = 38)				
IgA	27 (71)	5	9	10	3
IgG	16 (42)		2	6	8
IgM	1 (3)				1
	Histologically normal tissue from group 2 (n = 42)				
IgA	38 (91)	4	9	14	11
IgG	20 (48)			8	12
IgM	1 (2)				1

and the rest of the cycle. When the influence of the stage of the cycle was examined in parous and nulliparous women separately, however, cyclical changes were seen in the parous group but not in the nulliparae (table III). Sixteen parous women showed strong (+ + or + + +) synthesis in the luteal phase, compared with three in the proliferative phase ($P < 0{\cdot}05$). Among nulliparae there was no significant difference between the phases of the cycle. When patients in the luteal phase were examined synthesis was significantly greater among parous women than among nulliparae ($P < 0{\cdot}05$). In neither parous nor nulliparous women did the amount of IgA synthesis correlate with plasma oestradiol levels. There was no significant difference between the groups of parous and nulliparous women in average age, time since menarche, or plasma progesterone or oestradiol levels.

TABLE III—*Influence of parity and stage of menstrual cycle on in-vitro synthesis of IgA*

	Phase of menstrual cycle	No of positive specimens (and % of total)	Mean intensity of labelling of autoradiographs (No of specimens)			
			+ + +	+ +	+	±
Nulliparous patients (n = 22)	Proliferative (n = 12)	11 (92)	2	2	7	
	Luteal (n = 10)	6 (60)		1	4	1
Parous patients (n = 43)	Proliferative (n = 17)	12 (71)	1	2	5	4
	Luteal (n = 26)	23 (89)	6	10	3	4

Influence of oral contraceptives—Thirteen patients were taking oral contraceptives, and IgA synthesis was detected in 12 of them. This proportion (92%) was not significantly different from the overall total.

IMMUNOFLUORESCENT IDENTIFICATION OF IMMUNOGLOBULIN

IgA—Table IV shows the numbers of specimens that were positive

when sections were examined after staining with fluorescent antibody to IgA. Plasma cells were seen in 71% of the sections and the number of cells was moderate or large in 14 of the 24 positive specimens. In all but two of the sections the cells were seen in greater numbers in the intralobular stroma than in the extralobular stroma. Fig 2 shows a section graded as + + + for plasma cells. Deposits of IgA were seen in the lumina of ductules in 88% of the specimens and as non-cellular foci in the intralobular stroma in 53%. In only five specimens (15%) were deposits seen outside the lobules.

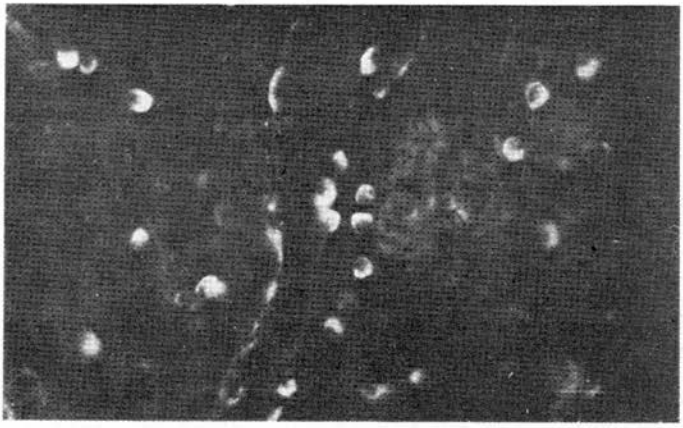

FIG 2—Section of breast lobule stained with FITC conjugated anti-IgA showing fluorescing plasma cells. (× 1910.)

TABLE IV—*Detection of IgA in 34 tissue specimens by immunofluorescence*

	No of positive specimens (and % of total)	Mean intensity of labelling of positive specimens (No of specimens)			
		+ + +	+ +	+	±
Plasma cells	24 (71)	4	10	5	5
Non-cellular material { Duct lumen	30 (88)	6	13	7	4
Non-cellular material { Lobule ..	18 (53)		2	13	3
Non-cellular material { Stroma ..	5 (15)	1		2	2

IgG—IgG-containing cells were seen in only three sections. Non-cellular deposits of IgG were seen in 10 sections (29%), and all were weakly fluorescent or barely visible (+ or ±). More deposits of IgG were seen in the extralobular stroma than in the lobules or the lumina of the ductules.

IgM—Fluorescent IgM-containing plasma cells were seen in one specimen and non-cellular deposits of IgM in three. The IgM deposits showed only barely detectable fluorescence.

Comparison of results classified according to parity, stage of cycle,

contraceptive use, and diagnosis showed no significant differences between groups in terms of numbers of positive sections or pattern of fluorescence. In those patients in the luteal phase of the cycle, more IgA-positive specimens were again found in the parous group (70%) than among the nulliparae (50%), but the difference was not significant.

The total number of specimens examined by immunofluorescence was limited to 34 because of technical problems or shortage of tissue. Also sections in which no lobular tissue could be identified were excluded. Despite this selection there was good overall agreement in the proportion of specimens found to be positive by the fluorescence and organ culture methods.

REPEATABILITY OF RESULTS

Four specimens were taken from different parts of a single breast at mammoplasty and coded as separate specimens. None of the observers knew which specimens came from the same patient. After organ culture IgA synthesis was graded as +++ in two and ++ in the other two; IgG synthesis as ++ in two and + in two; and IgM negative in all four. Immunofluorescence was done on three of these specimens. IgA-containing plasma cells were seen in two and graded as + and ±; non-cellular deposits of IgA in the intralobular stroma were graded as + in all three; and IgA deposits within the lumina of ductules were + in two and ± in one. Neither cells nor deposits of IgA were seen in the extralobular stroma in any of the three specimens. No cells or deposits were seen in the sections stained for IgG or IgM.

Discussion

IgA is the principal immunoglobulin in most human external secretions, including colostrum and milk. IgA in salivary and intestinal secretions is known to be synthesised locally by subepithelial plasma cells.[10] Many plasma cells are seen in the lactating human breast,[3] and this has led to the assumption that the IgA in human milk is also locally synthesised. Local synthesis of immunoglobulin by mammary tissue has been shown in several animal species,[11 12] but there is only one brief report of such a study in the human breast.[13]

Our observation that the non-lactating breast produces IgA rather than other immunoglobulins suggests that this synthesis is not a non-specific inflammatory response but a process associated with secretion. This interpretation is supported by the localisation of IgA in plasma cells associated with the lobules. Deposits of IgA are also concentrated in the lobules and particularly in the lumina of ductules, whereas IgG-positive cells are randomly distributed throughout the lobules and stroma.

Many of our biopsy specimens were taken from women who had breast nodules and were therefore not truly "normal." Mammoplasty specimens gave similar results, however, and we therefore conclude that IgA synthesis is not a reaction to benign breast disease but a property of normal breast tissue.

The secretion of IgA into the ductules may represent a form of defence against the entry of infection, but it seems more likely that the IgA secretion is simply a basal level of activity in an organ whose primary function is full lactation. In our primitive ancestors the breast was probably lactating almost continuously during the reproductive years,[11] and it is unlikely that mechanisms adapted specifically for the non-lactating state have had time to evolve.

IgA synthesis occurs in both parous and nulliparous patients, but the observation that cyclical changes in IgA synthesis occur only in parous women implies some functional difference in the breast after the first pregnancy. The increase in IgA synthesis in the luteal phase of the cycle among parous women suggests that after the first pregnancy the breast is more sensitive to progesterone. A study of DNA synthesis by human breast epithelium in vitro[15] has also shown cyclical variation in tissue from parous women and no cyclical variation among nulliparae.

The nulliparous breast is more susceptible to cancer, and the risk of subsequent breast cancer increases with increasing time elapsed between menarche and first pregnancy.[16 17] The suggestion in our results that the nulliparous breast is less responsive to progesterone than the parous breast may simply be a reflection of the "immaturity" of the nulliparous breast, but the difference in hormonal sensitivity of breast tissue may have a direct bearing on its susceptibility to malignant change. It has been suggested that "unopposed" oestrogen stimulation can lead to neoplastic changes[18]: if the nulliparous breast is relatively insensitive to progesterone this might mean that oestrogen stimulation during the menstrual cycle is inadequately "opposed" by progesterone.

We have no evidence that oral contraceptives produce abnormal stimulation of breast tissue. Our results suggest that the nulliparous breast will be relatively insensitive to the progestogen component of the combined oral contraceptive; the effect of the oestrogen component remains a matter for conjecture.

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For Debate . . .

Breast cancer, pregnancy, and the pill

JAMES OWEN DRIFE

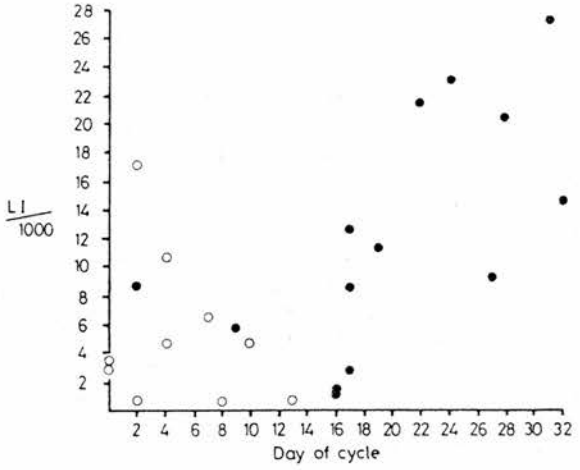
There is still no satisfactory explanation for the effect of pregnancy on the risk of breast cancer. Breast cancer is commoner among women who have never borne children than among parous women, and the longer a woman delays her first pregnancy the more she increases her risk of developing the disease.[1 2] Recently it has been suggested[3] that prolonged use of oral contraceptives before first full-term pregnancy may increase the risk of breast cancer, although after this pregnancy the risk is probably unaffected by taking the pill.

Clearly the first full-term pregnancy does something to alter a woman's risk of developing breast cancer. Before this pregnancy the breast is at greater risk of precancerous change: afterwards the risk diminishes. Whether or not a woman breast-feeds is probably irrelevant[4]: so far as the breast is concerned the important thing is that a full-term pregnancy has occurred. There are three possible explanations for this. One is that a woman's hormonal state is permanently altered by her first pregnancy. This has been investigated but no consistent answer has emerged.[5 6] A second possibility is that during the first pregnancy cells that have undergone precancerous change are destroyed, immunologically or otherwise. There is no evidence that this occurs, and indeed since pregnancy is a period of increased immunological tolerance such a phenomenon seems unlikely. The third explanation is that the breast epithelium itself is permanently changed by pregnancy. There is good evidence that such a change occurs.

Effect of parity on the breast

Early in pregnancy little change occurs in the breast lobules, but during the second trimester they undergo proliferation.[7] After pregnancy (or at the end of breast-feeding) involution occurs, until the histological structure of the breast is similar to that of a nulliparous woman's breast. Recently, however, it has been shown[8] that the epithelium of the ductules remains slightly but importantly altered: "clear" cells are less numerous after first pregnancy than in the ductules of nulliparous women. More striking than this histological change, however, is the physiological change in the epithelium, shown by its altered response to hormones.

Synthesis of DNA in vitro by specimens of normal breast tissue from parous women shows a clear cyclical pattern according to the day of the menstrual cycle on which tissue was obtained[9] (fig). Synthesis is maximum during the luteal phase of the cycle and increases rapidly with the appearance of progesterone in the circulation. By contrast, among nulliparous women no cyclical pattern is seen. In another study[10] in-vitro synthesis of immunoglobulins showed a similar phenomenon: among parous women there was a pronounced increase in IgA synthesis during the luteal phase of the cycle, but among nulliparous women no cyclical changes occurred.



In-vitro DNA synthesis by normal breast tissue from parous women: relationship between epithelial cell labelling indices and day of cycle. ○ denotes specimens from women with a plasma progesterone concentration under 1 ng/ml; ● denotes those where concentration was over 1 ng/ml, signifying presence of a corpus luteum. (From Masters *et al*[9] with permission.)

In both these studies the plasma concentrations of progesterone and oestrogen were similar in nulliparous and parous women. The difference between these two groups of women lay not in the state of their hormones but in the ability of the breast tissue to respond to these endogenous hormones. That is to say, pregnancy permanently altered the hormone responsiveness of the epithelium. How this effect is brought about is not clear: possibly the high progesterone concentrations of late pregnancy induce progesterone receptors. We can conclude, however, that after a single full-term pregnancy the breast epithelium becomes capable of responding to circulating progesterone at the relatively low concentrations present during the normal menstrual cycle. Until the first full-term pregnancy has occurred, it cannot respond to progesterone at these concentrations.

Carcinogenesis and the pill

Exposure to oestrogen "unopposed" by progesterone is now recognised as a carcinogenic influence on the endometrium.[11] In recent years regimens of so-called "hormone replacement therapy" for postmenopausal women have been altered by adding a progestogen in the hope that this will prevent the de-

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opment of endometrial carcinoma.[12] The incidence of breast ıcer has also increased among women receiving prolonged ıtment with unopposed oestrogen,[13] and so it is possible—but it no more strongly—that unopposed oestrogen has a simi- carcinogenic effect on the breast.[14]

The importance of an inadequate response to progesterone v becomes clear. If before first pregnancy the breast has dequate progesterone receptors and cannot respond to logenous progesterone, then during the normal cycle it will in ct be exposed to "unopposed" oestrogen—despite the pre- ce of normal concentrations of progesterone in the circulation. erefore it does not matter whether a young woman has ovu- ry cycles before her first pregnancy, because even if proges- one is present it will be unable to modify the effect of her logenous oestrogen. By contrast, after her first pregnancy the dence of anovulatory cycles matters a great deal,[15-17] since gesterone, if present, will now be capable of "opposing" the ct of oestrogen.

low does this hypothesis affect the risk to women who take l contraceptives? Firstly, it implies that the effects of the pill be different among nulliparous and parous women—as is being suggested by epidemiological surveys.[3 18 19] The bined oral contraceptive provides oestrogen balanced by gestogen, but if the breast of the nulliparous woman cannot ond to the progestogen component, then a young girl ng an oral contraceptive is effectively exposing her breast helium to "unopposed" oestrogen. On the other hand, by ng the pill she is suppressing the production of oestrogen by own ovaries. What we need to know is whether exposure to genous oestrogen at the steady low concentration provided he pill is any more dangerous than endogenous oestrogen ne higher fluctuating concentrations supplied by the ovary. s question has not yet been answered.

he hypothesis summarised here explains the importance of iparity and delayed first pregnancy as risk factors for subse- nt breast cancer. It also explains the importance of early arche,[2 20] since the sooner the menstrual cycles begin the ner the "unopposed" oestrogen begins to act on the breast nelium. (The importance of a late menopause is explained he fact that late in a woman's reproductive life her cycles are nly anovular,[21] and so a woman with a late menopause eriences more oestrogen-only cycles, although her breast nelium could respond to progesterone if it were present.) hypothesis could perhaps also explain the recent uncon- ed observation[3] that an early miscarriage before first full- pregnancy is another risk factor: concentrations of both rogen and progesterone increase in early pregnancy, but in ulliparous woman only the oestrogen will affect the breast. ne hypothesis does not attempt to explain the racial and raphical variations in the incidence of breast cancer.[22] does it offer an opinion on whether one particular oestrogen as oestradiol[23] or oestrone[24]) is less carcinogenic than an- r. What seems clear above all is that future study should con- ate less on women's hormonal profiles and more on the ts of the hormones on the breast itself.

is hypothesis is based on work done while I was an MRC clinical rch fellow at the MRC Unit of Reproductive Biology, Edinburgh.

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Does endometriosis occur more often in sterilised than in unsterilised women?

Endometriosis after salpingectomy or tubal sterilisation was first reported in 1930.[1] This has recently been confirmed[2]; the condition may also occur after tubal coagulation and may begin within a few months of operation. The endometriosis may be painful[2] and may spread several centimetres from the tubal stump.[1] The original recommendation for its prevention after salpingectomy was that hysterectomy should be performed in preference whenever possible,[1] but now it is thought that excision of a wedge of tubal uterine cornu is sufficient.[2] It is not clear, however, how to prevent its occurrence after other forms of tubal occlusion. The frequency of the condition after tubal sterilisation is unknown. In one study,[3] fistula formation with endometriosis was found in six out of 20 patients with failed sterilisation, and so a search was made for the condition among patients with successful sterilisation: microscopic evidence of endometriosis was found in 63% of patients with successful tubal cautery but in only 28% of patients with successful Pomeroy sterilisation. The process occurs within one to four years of operation, and the incidence is lower if the proximal tubal stump measures over 4 cm. It must be emphasised that in this study[3] endometriosis was specifically sought and was mainly microscopic. The risk of painful clinical endometriosis to a woman undergoing laparoscopic sterilisation with clips or rings is unknown, but must be very low indeed.

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